UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA

IDENTIFICATION OF THE REGULATORY MECHANISMS AND FACTORS INVOLVED IN THE BRAIN-SPECIFIC EXPRESSION OF *CYP46A1*

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IDENTIFICAÇÃO DOS MECANISMOS E FACTORES ENVOLVIDOS NA REGULAÇÃO DA EXPRESSÃO DO *CYP46A1* NO ENCÉFALO

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ABBREVIATIONS

24OHC	24(*S*)-hydroxycholesterol
27OHC	27-hydroxycholesterol
3xTgAD	triple transgenic mice of AD
ABC	ATP-binding cassette
ACAT	acyl-coenzyme A: cholesterol O-acyltransferase
AD	Alzheimer's disease
AICD	APP intracellular domain
APO	apolipoprotein
APP	amyloid precursor protein
Aβ	amyloid β peptide
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
bHLH	basic helix-loop-helix
BLAST	Basic Local Alignment Search Tool
BTD	buttonhead
DAC	5'-Aza-2'-deoxycytidine
DNA	deoxyribonucleic acid
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CNS	central nervous system
CRSP	co-factor required for Sp1 activation
CSF	cerebrospinal fluid
CTF	COOH-terminal fragment
CYP	cytochrome P450
DBD	DNA binding domain
DMEM	Dulbecco's modified Eagle's medium
DNMT	DNA methyltransferase
EMSA	electrophoretic mobility shift assay
EAAT2	excitatory amino-acid transporter 2
farnesyl-PP	farnesyl diphosphate

GC-MS	gas chromatography - mass spectrometry
HDAC	histone deacetylase
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzime A
ICC	immunocytochemistry
KLF	Krüppel-like factors
LDLr	low-density lipoprotein receptors
LRP	lipoprotein receptor-related protein
LTP	long-term potentiation
LXR	liver X receptor
MAP-2	microtubule-associated protein 2
MBD	methyl-CpG binding domain
mRNA	messenger RNA
NeuroD	neurogenic differentiation factor
NF200	neurofilament 200
NRSF	neuron-restrictive silencer factor
qPCR	quantitative real-time polymerase chain reaction
NT2	Ntera2/clone D1
PDL-MG	poly-D-lysine and Matrigel
PTM	post translational modification
RA	retinoic acid
RE 1	repressor element 1
REST	RE 1-silencing transcription factor
RNA pol II	RNA polymerase II
SAM	S-adenosyl methionine
SL2	Schneider cell line
Sp	specificity protein
SREBP	sterol-regulatory element binding protein
TAD	transactivation domain
TAFs	TBP-associated factors
TBF	TATA binding protein
TFs	transcription factors
TSA	trichostatin A
TUJ-1	type III β-tubulin
WT	wild type

ABSTRACT

Cholesterol has a crucial role in central nervous system physiology and cell signaling, and many studies correlate alterations in brain cholesterol homeostasis with neurodegenerative diseases. The *CYP46A1* gene codes for the cholesterol 24-hydroxylase (CYP46A1), a cytochrome P450 specifically expressed in neurons, that is responsible for the majority of brain cholesterol turnover. However, despite its physiological importance, the molecular mechanisms underlying the human *CYP46A1* expression had not been characterized. Therefore, our work aimed to identify regulatory elements and factors involved the *CYP46A1* brain-specific expression, and further assess if *CYP46A1* transcription is regulated by epigenetic modifications, such as DNA methylation. Moreover, we aimed to identify a human cell model that could be a valuable tool for the study of cholesterol homeostasis in human neurons.

In our initial studies, we cloned and characterized the human *CYP46A1* promoter. Functional deletion analysis, over-expression studies, site-directed mutagenesis and gel-shift assays identified that not only Sp transcription factors control *CYP46A1* transcription, but are most probably responsible for cell type specificity. To test our hypothesis that an increase in the (Sp3+Sp4)/ Sp1 ratio would result in *CYP46A1* transcriptional activation, we differentiated Ntera2/clone D1 (NT2) human teratocarcinoma cells into post-mitotic neurons (NT2N). We demonstrated for the first time a significant increase in CYP46A1 mRNA and protein levels in a human cell culture, and identified a concomitant decrease in the levels of Sp1 associated with the proximal promoter of this gene. Nevertheless, we did not observe any conserved pattern in Sp protein binding to other Sp-regulated gene promoters, suggesting that Sp-DNA binding and transcriptional activity is highly dependent on the neuronal chromatin context. Moreover, we showed that throughout NT2 differentiation, HMG-CoA

synthase, HMG-CoA reductase, SREBP2 and LDLr, key players in brain cholesterol homeostasis, present expression profiles that mimic what is thought to occur *in vivo*. Our results also suggest that progenitor cells eliminate cholesterol in the form of 27-hydroxycholesterol while neurogenesis induces a shift to the 24-hydroxylase-dependent elimination pathway. Finally, we showed that the demethylating agent 5'-Aza-2'-deoxycytidine (DAC) is a *CYP46A1* inducer. Surprisingly, bisulfite sequencing analysis revealed that the *CYP46A1* core promoter is completely unmethylated in both human brain and non-neuronal human tissues where CYP46A1 is not expressed. We demonstrated that DAC induces *CYP46A1* expression, in a DNA methylation-independent mechanism, by decreasing Sp3/HDAC binding to the proximal promoter.

Collectively, our results provide new insights on the regulatory circuits that control *CYP46A1* transcription, and contribute to the identification of potential therapeutic approaches that can modulate *CYP46A1* expression.

Keywords: *CYP46A1* - Gene regulation - Sp proteins - Brain cholesterol - Alzheimer's disease

RESUMO

O colesterol é o principal constituinte da mielina, sendo essencial para a estrutura e função do sistema nervoso central (CNS). Devido à eficiência da barreira hemato-encefálica, não existem trocas de colesterol entre as células do encéfalo e a circulação sistémica, sendo o metabolismo do colesterol no CNS independente do metabolismo deste esterol no resto do organismo. O colesterol necessário ao bom funcionamento do CNS é assim produzido *in situ*, e a sua homeostasia é mantida por um conjunto de processos interdependentes, como a síntese, utilização e armazenamento, transporte e excreção.

Recentemente, foi descrita a principal via de eliminação do colesterol do encéfalo, que consiste na sua conversão no derivado 24(*S*)-hidroxicolesterol (24OHC), um metabolito mais polar que, ao contrário do colesterol, tem a capacidade de atravessar a barreira hemato-encefálica. Uma vez na corrente sanguínea este esterol é transportado para o fígado, onde pode ser novamente metabolizado, e finalmente eliminado do organismo. Estima-se que esta via de eliminação seja responsável por cerca de dois terços da excreção do colesterol em excesso do encéfalo. O enzima responsável pela conversão do colesterol em 24OHC, o CYP46A1, pertence à superfamília dos citocromos P450 e é exclusivamente expresso nos neurónios. Apesar da sua importância, os mecanismos moleculares subjacentes à expressão específica de tecido do *CYP46A1* ainda estão pouco explorados, desconhecendo-se se a transcrição deste gene é modulada por sinais endógenos ou exógenos. Como tal, o trabalho aqui apresentado teve como objectivos a identificação dos elementos e factores de transcrição envolvidos na expressão basal e específica de tecido do *CYP46A1*, nomeadamente o papel específico da família de factores de transcrição Sp, bem como a

identificação de um modelo celular humano que permitisse, em particular, estudos de regulação da expressão do *CYP46A1*, e de um modo mais geral, a compreensão dos mecanismos celulares de regulação da homeostasia do colesterol em neurónios pós-mitóticos. Finalmente caracterizámos igualmente o papel da metilação do DNA, na expressão específica de neurónios do *CYP46A1*.

Na primeira fase do trabalho clonámos e caracterizámos a região 5' adjacente do gene *CYP46A1* humano. A análise funcional do promotor permitiu identificar a região -236/-64, como sendo indispensável para a expressão basal deste gene. Uma vez que uma análise bioinformática revelou a presença, no promotor, de dez potenciais sítios de ligação para os factores de transcrição da família Sp, fomos investigar o papel destas proteínas na regulação da transcrição do *CYP46A1*. Ensaios de sobre-expressão, mutagénese dirigida e ensaios do desvio de mobilidade electroforética (EMSA), permitiram não só identificar os factores de transcrição da família Sp, como responsáveis pela actividade basal do promotor *CYP46A1*, bem como sugerir que a ligação preferencial das proteínas Sp3 e Sp4 seria responsável pela expressão específica nos neurónios. De facto, a cotransfecção de vectores de expressão que codificam para Sp3 e Sp4 promovem uma transactivação elevada do promotor, sendo igualmente estas proteínas que se ligam aos elementos de resposta Sp presentes no promotor próximo do *CYP46A1*, quando são usados, em ensaios de EMSA, extractos nucleares preparados a partir de neurónios corticais de rato. Estes resultados reforçaram o papel destas proteínas na regulação do *CYP46A1*, e levaram-nos a colocar a hipótese de que um aumento na razão das proteínas (Sp3+Sp4)/ Sp1 ligadas ao promotor próximo do gene poderia ser responsável pela expressão específica de tecido.

Na tentativa de identificar um bom modelo celular humano para estudos de regulação da expressão do *CYP46A1*, procedemos à diferenciação de células de teratocarcinoma embrionário, Ntera2/clone D1 (NT2). Após tratamento de longa duração com ácido retinóico, estas células têm capacidade de diferenciar em neurónios pós-mitóticos. Verificámos uma indução significativa nos níveis de mRNA e proteína CYP46A1 nas fases finais da diferenciação, concomitante com uma diminuição nos níveis proteicos de Sp1. Através de ensaios de imunoprecipitação da cromatina (ChIP) pudemos avaliar o recrutamento das proteínas Sp para o promotor próximo do *CYP46A1*, tendo sido detectada uma diminuição da ligação do factor de transcrição Sp1, após a diferenciação, mantendo-se associados ao promotor níveis idênticos de Sp3 e

Sp4. Assim, com este modelo celular foi-nos possível confirmar os resultados obtidos *in vitro*, de que as proteínas Sp3 e Sp4 são os principais factores de transcrição ligados ao promotor próximo do *CYP46A1*, em neurónios pós-mitóticos. Estudámos ainda a ligação das proteínas Sp às regiões promotoras dos genes reelina, MOR, GRIN1 e GRIN2, que se exprimem em neurónios, e que previamente foram descritos como sendo regulados por estes factores de transcrição. Estes estudos permitiram-nos aferir que não parece existir um padrão na alteração da ligação das proteínas Sp às regiões promotoras de genes de expressão neuronal, sugerindo que, tal como descrito para outros tipos celulares, a ligação das proteínas Sp ao DNA depende do contexto estrutural da cromatina em cada região promotora específica.

Alterações na homeostasia do colesterol no CNS têm sido associadas a diversas doenças neurodegenerativas, nomeadamente à doença de Alzheimer. No entanto, pouco se sabe sobre os mecanismos que regulam a metabolização do colesterol nos neurónios. Isto deve-se em parte ao facto de, até à data, não se conhecer um modelo celular em que o gene *CYP46A1* fosse expresso em níveis elevados. Como tal, fomos caracterizar o perfil de expressão de genes que codificam para proteínas envolvidas na síntese, transporte e catabolismo do colesterol, bem como medir os níveis de oxisterois, produtos do catabolismo do colesterol, ao longo da diferenciação das células NT2. Os nossos resultados demonstraram que os principais intervenientes na manutenção da homeostasia do colesterol no encéfalo são expressos nas NT2 diferenciadas, exibindo um perfil semelhante ao que se pensa existir *in vivo*, nomeadamente, verificámos uma diminuição dos níveis de expressão dos enzimas HMG-CoA sintase e HMG-CoA reductase. Para além disso, pudemos verificar que parece existir nos neurónios pós-mitóticos uma substituição da via de catabolismo dependente do enzima CYP27A1, responsável pela hidroxilação do colesterol na posição 27, para a via da hidroxilação do colesterol na posição 24. Os nossos resultados vieram realçar a importância deste modelo celular para estudos futuros sobre a homeostasia do colesterol em neurónios pós-mitóticos.

Uma vez que inicialmente verificámos que o promotor do *CYP46A1* é muito rico em dinucleótidos guanina-citosina (CpG) e está localizado numa ilha CpG, na parte final deste trabalho, investigámos o papel da metilação do DNA na regulação da expressão deste gene. Estes estudos demonstraram que o fármaco hipometilante 5'-aza-2'deoxicitidina (DAC) leva a um aumento na expressão do *CYP46A1*, de um

modo independente da desmetilação do promotor próximo, e que o pré-tratamento de células de neuroblastoma humano, SH-SY5Y, com este fármaco, potencia o efeito activador da expressão do CYP46A1, induzido pelo inibidor das desacetilases de histonas – tricostatina A. De facto, após tratamento do DNA genómico com bissulfito de sódio, verificámos por sequenciação, que o promotor próximo deste gene se apresenta totalmente desmetilado, tanto em tecidos onde este se exprime a níveis elevados, como em tecidos onde o mRNA CYP46A1 não é detectado. Através da utilização de ensaios de western blot, EMSA e ChIP, pudemos identificar que o tratamento com DAC induz uma diminuição dos níveis das proteínas Sp1 e Sp3, e uma consequente dissociação das proteínas Sp3 e das desacetilases de histonas 1 e 2 do promotor próximo do *CYP46A1*, concomitante com o aumento da expressão deste gene.

Em suma, com a realização deste trabalho contribuímos para a caracterização de importantes mecanismos moleculares que controlam a expressão do *CYP46A1*, através da identificação da relevância dos factores de transcrição da família Sp, tanto na regulação basal deste gene como na resposta ao agente desmetilante DAC. Por outro lado, este trabalho possibilitou ainda a identificação de um modelo celular humano para o estudo, não só da regulação da transcrição do *CYP46A1*, mas também do metabolismo do colesterol nos neurónios. A descoberta recente de que a sobre-expressão do CYP46A1 pode modular os níveis do péptido β-amilóide, aliada ao conhecimento de que o aumento dos níveis de 24OHC favorece o processamento não amiloidogénico da proteína percursora do péptido β-amilóide, pode ter implicações importantes em estratégias terapêuticas futuras que minimizem os danos e a progressão da doença de Alzheimer. A caracterização dos mecanismos de regulação do *CYP46A1* possibilitam ainda o desenvolvimento de possíveis abordagens terapêuticas que possam igualmente modular a expressão deste importante enzima do catabolismo do colesterol noutras patologias neurodegenerativas.

Palavras-chave: Metabolismo do colesterol no encéfalo - *CYP46A1* - Regulação da transcrição - Factores de transcrição Sp - Metilação do DNA

GENERAL INTRODUCTION AND OBJECTIVES

1.1 Brain Cholesterol

Cholesterol is essential for the proper functioning of eukaryotic cells, and in the central nervous system (CNS) it plays an essential role in myelination (Saher *et al.* 2005), stabilization and organization of axonal microtubules (Fan *et al.* 2001), dendritic differentiation (Fan *et al.* 2002) and synaptic plasticity (Mauch *et al.* 2001). Since cholesterol plays a pivotal role, there seems to be a physiological imperative to maintain constant levels of this important molecule in the CNS (Bjorkhem *et al.* 2009).

Outside the CNS, cholesterol homeostasis is maintained by extremely precise regulation and coordination of uptake, synthesis and esterification. The cellular requirements for this sterol are covered by *de novo* synthesis and by the uptake of dietary cholesterol under the form of lipoprotein-cholesterol complexes from blood circulation (Martin *et al.* 2010). However, cholesterol metabolism in the CNS is distinct from that in other tissues (Dietschy & Turley 2004). No uptake of cholesterol from the plasma to the brain occurs, as the blood-brain barrier (BBB) prevents the diffusion of large molecules at the tight junctions between brain capillary endothelial cells (Bjorkhem & Meaney 2004). Two recent studies suggest the existence of mechanisms that can facilitate cholesterol uptake from the plasma to the brain, which raises the possibility that brain cholesterol metabolism is not completely isolated from the rest of the organism (Karasinska *et al.* 2009, Saito *et al.* 2009). However, these two studies rely on the brain-specific ablation of key enzymes in cholesterol synthesis (squalene synthase) and transport (ATP-Binding cassette A1 - ABCA1), not reflecting normal brain metabolism. Therefore, virtually all the cholesterol necessary to the good functioning of the CNS is thought to be synthesized *in situ* (Dietschy & Turley 2004).

1.1.1 Brain cholesterol synthesis

The CNS cholesterol is believed to reside in two different pools, the plasma membranes of neurons and glial cells and the myelin sheaths. Myelin represents up to half of the white matter and since it has been estimated that approximately 70% of all the cholesterol in the brain is associated with myelin, it is not surprising that the brain is the most cholesterol rich organ in the body (Bjorkhem & Meaney 2004).

During brain development, it is thought that most of the cholesterol synthesis occurs in oligodendrocytes associated with myelin production. As the CNS matures and myelin

production decreases, the pool of cholesterol in the CNS reaches a constant value. The rate of cholesterol synthesis declines and mainly reflects synthesis in the glial cell and neuron compartments (Dietschy & Turley 2004). The cholesterol biosynthetic pathway requires more than 20 dedicated enzymes distributed in different organelles, of which the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, that catalyzes the formation of mevalonate, is the major rate-limiting enzyme of this pathway (Brown & Goldstein 1980). HMG-CoA reductase activity, which is inhibited by statins, is subject to complex regulation, such as the feedback inhibition by high cholesterol through transcriptional control via the sterol-regulated element binding protein (SREBP) (Goldstein & Brown 1990). This is an energetically expensive and complex pathway that has a very elevated cost to the cell (Figure 1.1).

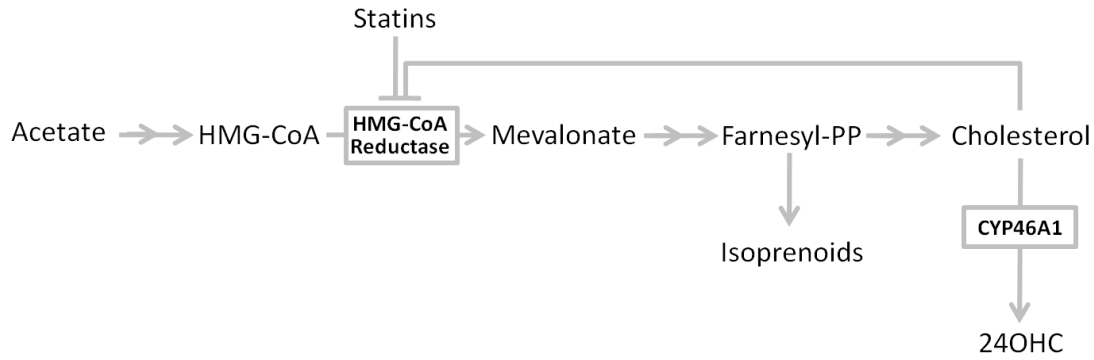

Figure 1.1. Cholesterol pathways in the brain. An abbreviated cholesterol biosynthetic pathway is shown where the key enzymes and intermediates are depicted. Double arrows imply multiple reactions. Statins reduce the flow of intermediates through the biosynthetic pathway by inhibiting HMG-CoA reductase, which is also inhibited by a negative feedback pathway activated by cholesterol. Farnesyl diphosphates (farnesyl-PP) are polyisoprenoid end products of the pathway. Catabolism of brain cholesterol is mediated by CYP46A1, which converts excess cholesterol into 24*S*-hydroxycholesterol (24OHC). *Adapted from Russell et al. 2009.*

Supported by the knowledge that astrocytes play important roles in the CNS, such as metabolic support for neurons, neuronal survival and differentiation, neuronal guidance, neurite outgrowth and synaptogenesis (Verkhratsky & Parpura 2010), and the fact that neurons are highly specialized cells responsible for the generation and exchange of chemical and electrical signals, Frank Pfrieger (2003) has put forward the hypothesis of a cholesterol shuttle between astrocytes and neurons. This hypothesis states that mature neurons rely mostly on cholesterol produced by astrocytes, which allows neurons to

dispense the energetically expensive cholesterol synthesis and focus on chemical signaling and the generation of electrical activity (Pfrieger 2003).

Several studies support the cholesterol shuttle hypothesis. It has been demonstrated that purified retinal ganglion cells exhibit little synaptic activity, unless they are co-cultured with astrocytes (Pfrieger & Barres 1997). Furthermore, two studies showed that astrocytes increase the number of mature and functional synapses of neurons by sevenfold and are required for synaptic maintenance *in vitro* (Nagler *et al.* 2001, Ullian *et al.* 2001). In these studies, a soluble glia-derived signal was found to induce synapse formation, and this was later identified as the apolipoprotein E (apoE). However, recombinant apoE failed to induce synapse formation in neurons. Since apoE containing lipoproteins are cholesterol carriers, Mauch and co-workers (2001) added cholesterol to the cultured neurons and demonstrated that cholesterol by itself induced almost the same number of synapses as those induced by astrocyte-conditioned medium. These results demonstrated that, astrocytes provide neurons with cholesterol in the form of apoE containing lipoproteins (Mauch *et al.* 2001). Furthermore, Fünfschilling and co-workers (2007) have disrupted the mouse squalene synthase gene, that codes for an enzyme critical for cholesterol synthesis, in cerebellar granule cells and in precerebellar nuclei in the adult mice brain. These mice had no histological signs of neuronal degeneration, displayed normal synapses ultrastructures, and exhibited normal motor coordination. These data imply that at least some populations of adult neurons do not require autonomous cholesterol synthesis for survival or function (Funfschilling *et al.* 2007). More recently, the Pfrieger laboratory has demonstrated that neurons show distinct profiles of biosynthetic enzymes, precursors and cholesterol metabolites when compared with astrocytes. Moreover, neurons have a less efficient cholesterol synthesis than astrocytes and there is massive down-regulation in the levels of neuronal intermediaries of the cholesterol biosynthetic pathway when neurons are maintained in co-culture with glial cells (Nieweg *et al.* 2009). These studies support the hypothesis that neurons reduce cholesterol synthesis and rely on delivery by astrocytes.

In contrast, there are also several evidences of cholesterol production in specific neuronal populations throughout the adult brain. Indeed, the first and last enzymes in the cholesterol biosynthetic pathway, HMG-CoA reductase and 7- dehydrocholesterol reductase, respectively, are expressed in neurons throughout the adult mouse brain and are particularly prominent in cortical, hippocampal, and cholinergic neurons (Korade *et al.* 2007). The analysis of 25 genes involved in cholesterol homeostasis from *in situ* data

provided by the Allen Brain Mouse Atlas, has revealed that the expression of cholesterol homeostasis genes is preferentially targeted to neuronal hippocampal pyramidal and granule layers (Valdez *et al.* 2010). In addition, the sterol-sensing transcription factors SREBP-1 and SREBP-2 are expressed in cortical and hippocampal neurons in the normal brain (Ong *et al.* 2000, Kim & Ong 2009). Furthermore, it has been shown that the brain-derived neurotrophic factor (BDNF) elicits transcription of enzymes in the cholesterol biosynthetic pathway in cultured cortical and hippocampal neurons, but not glial cells (Suzuki *et al.* 2007). Moreover, it has been shown that important intermediaries of the cholesterol biosynthetic pathway, like isoprenoids and ubiquinone are crucial for proper neuronal functioning (Maltese 1990, Crane 2001). These studies show that at least some cholesterol synthesis can occur in specific neuron populations.

Taken together, current data suggest that due to the structural and functional specialization of the different brain regions it is likely that some neurons may be autonomous while others may depend entirely on an external cholesterol supply (Pfrieger 2003).

1.1.2 Brain cholesterol transport, utilization and storage

Cholesterol metabolism and distribution are not uniform across all cell types present in the brain, thus an efficient system is necessary for the transport of cholesterol between the different cells of the CNS.

ApoE is present in the CNS at high levels and throughout development this apolipoprotein is responsible for the mobilization and redistribution of cholesterol in myelin sheaths and neuronal membranes, during growth, maintenance and repair (Leduc *et al.* 2010). ApoE is required for the uptake of lipoproteins into cells via low-density lipoprotein receptors (LDLr) under non-pathological conditions. Other lipoproteins, such as apoD and apoJ, and several receptors from the LDLr family are also expressed in the CNS (Martins *et al.* 2009), as well as some members of the ABC transporter family, like ABCA1 and ABCG1. These transporters are responsible for the lipid efflux from cells to apolipoproteins, and it has been shown that ABCG1 mediates the efflux of cholesterol from glial cells to partially lipidated apoE (Karten *et al.* 2006). It is also known that ligand activation of the liver X receptors (LXRs) by oxysterols, can elicit the expression of both *APOE* and *ABCA1* (Vaya & Schipper 2007), therefore promoting cholesterol transport between different cells in the brain. Indeed, the cholesterol transporter ABCA1

was recently shown to be a key regulator of cholesterol metabolism, since its ablation not only reduced the number of synapses and synaptic vesicles, but it also led to an increase in cholesterol uptake from the plasma to the brain, via a yet unknown mechanism, in order to compensate for the reduced brain cholesterol levels in these animals (Karasinska *et al.* 2009, Saito *et al.* 2009). However, the role of all of these lipoproteins and receptors/ transporters in the CNS is not well established, although they have been implicated in brain cholesterol homeostasis.

When a cell is not capable of autonomous cholesterol production, it relies on the uptake of cholesterol from the extracellular milieu. After internalization of the apoE containing lipoproteins, endossomes are formed and subsequently fused with lysosomes. Here the apoE-phospholipid-complexed cholesterol is released intracellularly as unesterified (free) cholesterol (Poirier 2003). In fact, most brain cholesterol is unesterified and is found within the myelin sheaths, whereas the remaining unesterified cholesterol is found in neurons, glial cells and extracellular lipoproteins (Puglielli *et al.* 2003). Free cholesterol can serve as a signaling molecule, for example inhibiting HMG-CoA reductase activity (Nakanishi *et al.* 1988), can be a precursor of steroid hormones (Baulieu 1998), can be incorporated into different cellular compartments and, together with sphingolipids, can form lipid rafts (Simons & Gerl 2010). Furthermore, cholesterol can be esterified by acyl-coenzyme A: cholesterol O-acyltransferase (ACAT) to be efficiently stored in the form of cholesterol esters (Shobab *et al.* 2005). This intracellular cholesterol reservoir serves as a readily available source of this sterol to be used in membrane remodeling associated with synaptic turnover and dentritic reorganization (Mauch *et al.* 2001).

1.1.3 Brain cholesterol efflux

As previously mentioned, cholesterol synthesis is very low in the adult state and this can be explained by a very efficient recycling of cholesterol in the CNS. In fact, brain cholesterol has an extremely long half-life that has been estimated to be of at least 5 years (Bjorkhem *et al.* 1998), whereas in plasma the cholesterol half-life has been estimated to be of approximately 2 to 4 months (Chobanian *et al.* 1962). Cholesterol homeostasis is a dynamic process and the continuous turnover of cholesterol in neurons may account for their ability to efficiently and quickly balance cholesterol levels required for dynamic structural changes such as neurite extension and synapse formation (Pfrieger 2003).

Although the brain cholesterol recycling processes are highly effective, there is some excretion of excess cholesterol. To date two different mechanisms of cholesterol excretion from the brain have been identified. The first mechanism described relies on a reverse cholesterol pathway. Through this pathway, cholesterol-laden apoE-containing lipoproteins pass into the cerebrospinal fluid (CSF) and from here into the plasma, thus transporting excess cholesterol from the brain into circulation (Pitas *et al.* 1987). However, this mechanism accounts for only a small percentage of cholesterol export from the brain, having been calculated to correspond to a flux of approximately 1 mg/ 24 h (Bjorkhem 2006).

If the apoE-dependent excretion was the only mechanism of elimination from the brain, cholesterol half-life would be of about 50 years (Bjorkhem 2006). In fact, the major pathway by which excess cholesterol is eliminated from the brain involves the conversion of cholesterol into 24(*S*)-hydroxycholesterol (24OHC). The enzyme responsible for this conversion was found to be a brain-specific cytochrome P450, the cholesterol 24-hydroxylase (CYP46A1) (Lund *et al.* 1999).

Lütjohann and co-workers determined that the brain contains about 80% of all 24OHC in the body (Lütjohann *et al.* 1996). By measuring the levels of this oxysterol in serum samples from the internal jugular vein and the brachial artery of several healthy volunteers, Bjorkhem and co-workers (1998) demonstrated that 24OHC is the major oxysterol transported from the brain into circulation. Furthermore, this study demonstrated that most of the 24OHC present in human circulation is of cerebral origin and that there is a net flux of this oxysterol of about 6.4 mg/ 24 h from the brain into the circulation (Bjorkhem *et al.* 1998).

Less than 1% of the total 24OHC present in circulation corresponds to a primary secretion of this oxysterol into the CSF (Leoni *et al.* 2004). More than 99% of 24OHC leaves the brain directly through the BBB, which implies an efficient transport of 24OHC through the BBB. In fact, 24OHC is a more polar molecule than cholesterol, which should make it less able to pass a lipophilic barrier, however the hydroxyl group added to the side-chain of the cholesterol molecule leads to a reordering of membrane phospholipids such that it is more favorable to exclude the oxysterol molecule (Theunissen *et al.* 1986, Meaney *et al.* 2002). In addition, it has been demonstrated that 24OHC passes the BBB at a much faster rate than cholesterol (Lütjohann *et al.* 1996, Bjorkhem *et al.* 1997).

The conversion of cholesterol into 24OHC has been calculated to be responsible for the excretion of approximately two thirds of all the cholesterol secreted from the brain. After entering circulation, 24OHC is metabolized in the liver, and finally eliminated from the organism (Bjorkhem *et al.* 2001).

It is thus evident that cholesterol homeostasis in the brain is maintained by a cycle of interdependent processes that include synthesis, utilization/ storage, transport and excretion (Figure 1.2).

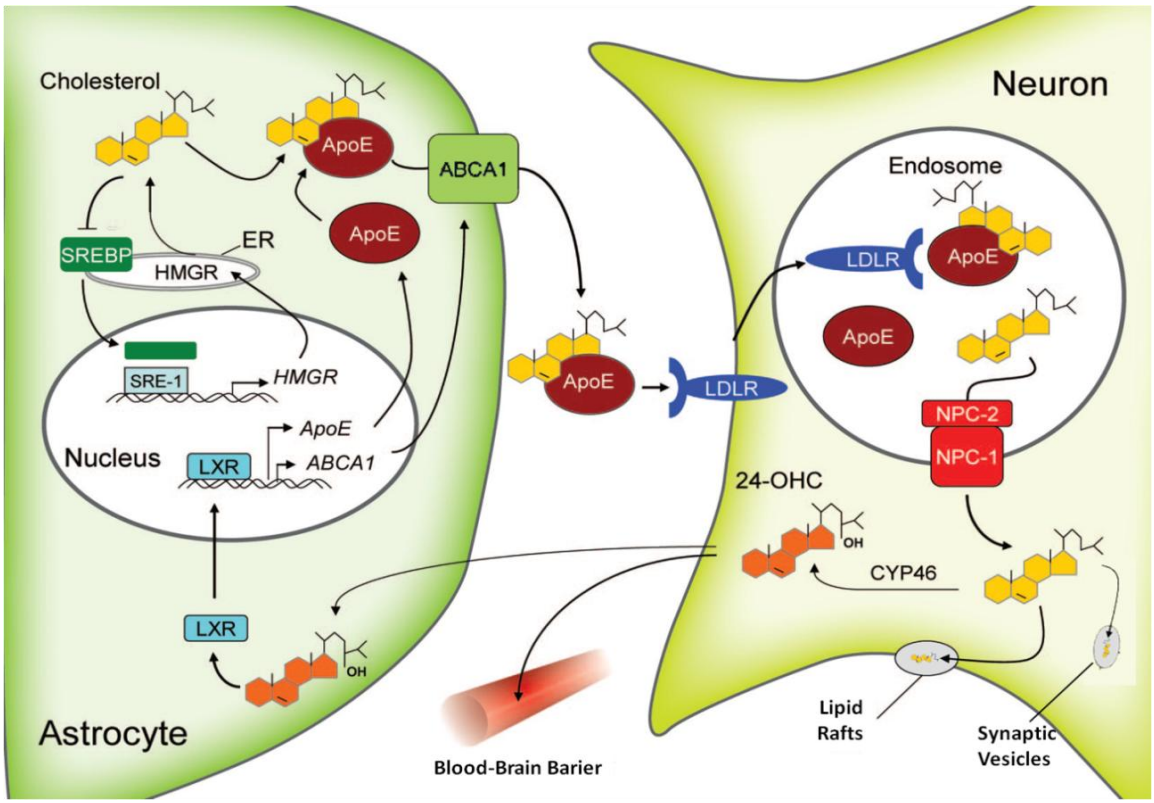

Figure 1.2. Cholesterol shuttle from astrocytes to neurons. It is hypothesized that, in the adult brain, neurons rely on delivery of cholesterol produced by astrocytes. Cholesterol is synthesized in astrocytes, delivered by lipoprotein particles to neurons, where it can be integrated in lipid rafts and can be incorporated into synaptic vesicles. Excess cholesterol in neurons is disposed of by conversion into 24OHC, this and other oxysterols can cross the blood–brain barrier, these sterols are important ligands of LXR, which translocate to the nucleus and induce expression of several genes important for cholesterol homeostasis in astrocytes. *Adapted from Benarroch 2008.*

1.1.4 Oxysterols in the brain

As previously mentioned, cholesterol destined to be eliminated from the brain is mainly oxidized at the 24 position to produce 24OHC. However, in a number of extra-

hepatic organs and tissues excess cellular cholesterol is oxidized at the 27 position originating 27-hydroxycholesterol (27OHC), transported in the circulation and eliminated (Heverin *et al.* 2005). Due to the fact that 27OHC is structurally very similar to 24OHC, it can pass the BBB and enter the brain, where it is rapidly metabolized having low steady-state levels (Heverin *et al.* 2005, Meaney *et al.* 2007). Interestingly, there is a flux of 27OHC from the plasma to the brain, in the opposite route to the flux of 24OHC. The implications of such a flux will be discussed in section 1.1.5.4.

Both 24OHC and 27OHC are ligands for LXRs (Lehmann *et al.* 1997, Fu *et al.* 2001). LXRs are nuclear hormone receptors activated by oxysterols that act as cholesterol sensors and respond to elevated sterol levels, modulating the expression of their target genes. Ligand activation of LXRs promotes cholesterol efflux from glia and primary neurons (Whitney *et al.* 2002, Koldamova *et al.* 2003, Abildayeva *et al.* 2006), and ablation of either LXRα or LXRβ in a mouse model of Alzheimer's Disease (AD) results in increased senile plaque load (Zelcer *et al.* 2007). Furthermore, activated LXRs have been shown to increase the expression of several genes involved in brain cholesterol homeostasis, such as *APOE* (Abildayeva *et al.* 2006), *ABCA1* (Liang *et al.* 2004) and the *LXRα* gene itself (Laffitte *et al.* 2001). These data point to the importance of both 24OHC and 27OHC in the maintenance of brain cholesterol homeostasis by acting as ligands for LXRs.

1.1.5 Brain cholesterol homeostasis disruption

The interdependent processes that regulate brain cholesterol homeostasis have to be precisely regulated for the good functioning of the brain. Actually, several studies point to a link between disruption of brain cholesterol homeostasis and the development of several neurological diseases, including AD, Huntington's and Parkinson's disease and Niemman-Pick type C disease (Martin *et al.* 2010, Karten *et al.* 2009, Liu *et al.* 2010, Block *et al.* 2010).

Several studies have already provided links between altered cholesterol homeostasis and AD. Indeed, epidemiological studies have revealed that the *APOE* ε4 allele is the major genetic risk factor in sporadic AD (Corder *et al.* 1993). Moreover, Notkola and co-workers evaluated the relation between *APOE* ε4 allele and plasma cholesterol levels on the risk of developing AD and firstly pointed to the possibility that high levels of serum total cholesterol could be an independent risk factor for this disease (Notkola *et al.* 1998).

Later that year, this hypothesis was corroborated by another group that demonstrated for the first time that high levels of total serum cholesterol predominantly in the form of LDL influence the AD-related pathology (Kuo *et al.* 1998). A significant number of studies point to a decreased susceptibility to AD in patients treated with statins, a class of cholesterol lowering drugs that inhibit HMG-CoA reductase (Jick *et al.* 2000, Hajjar *et al.* 2002). In the last years an effort has been made to understand how high plasma cholesterol, which is independent of brain cholesterol metabolism, can increase the risk to develop AD. Moreover, several studies endeavored to elucidate the molecular mechanisms by which disruption of brain cholesterol homeostasis alters the risk associated with AD.

AD is the most common neurodegenerative disease, defined clinically as a slow progressive loss of cognitive functions that leads to dementia and death. It is characterized by the aggregation and deposition of misfolded proteins, in particular aggregated amyloid β peptide (Aβ) in the form of extracellular senile plaques, and hyperphosphorlylated tau protein in the form of intracellular neurofibrillary tangles, which ultimately result in massive neuronal death and cognitive impairment (Bertram *et al.* 2010).

The amyloid cascade hypothesis states that deposition of Aβ, the main component of the senile plaques, is the causative agent of AD and that the neurofibrillary tangles, cell loss, vascular damage, and dementia follow as a direct result of this deposition (Hardy & Higgins 1992). This hypothesis has been updated to include the knowledge that senile plaques serve as a deposit for the toxic Aβ and are not the toxic agents themselves (Hardy 2006). Although several studies contradict the specific sequence of events leading to the production of Aβ, as critical in the initiation of AD pathogenesis (reviewed in Korczyn 2008) there is indisputable evidence that amyloid precursor protein (APP) processing is clearly an important event in AD.

1.1.5.1 Cholesterol and APP processing

The α-, β- and γ- secretases are responsible for the proteolytic cleavage of APP. Two major pathways of APP cleavage have been characterized (Figure 1.3): the protein may be cleaved by the α- secretase, which prevents the formation of Aβ and results in the production of a secreted fragment (αAPP) and a shorter COOH-terminal fragment (αCTF - C83) that is further cleaved by γ- secretase, giving rise to the p3 peptide and to the APP

intracellular domain (AICD). Alternatively, APP can be proteolysed by β- secretase, giving rise to a secreted fragment (βAPP) and a βCTF (C99), this then is cleaved by γ-secretase originating the AICD and the deleterious Aβ, which is released to the extracellular milieu (Selkoe 2001).

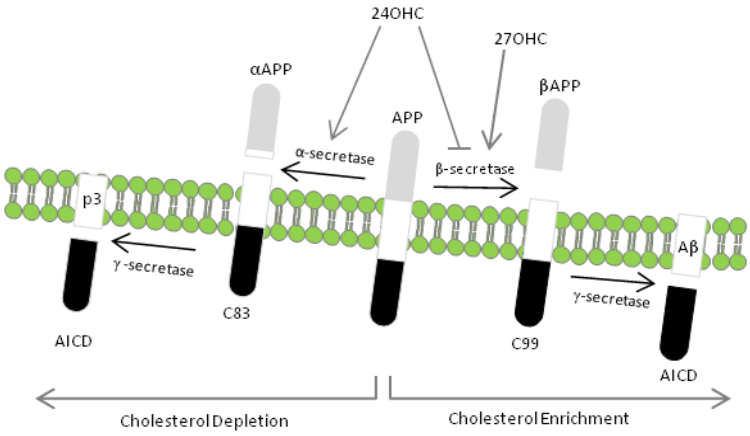

Figure 1.3. APP processing. APP is first cleaved by α- or β- secretase, resulting in the shedding of nearly the entire ectodomain (αAPP and βAPP) and the generation of membrane bound α-CTF (C83) or β-CTF (C99). These are subsequently cleaved by γ- secretase to release p3 or Aβ peptides, respectively. γ- secretase also generates a cytoplasmic polypeptide, the AICD. The levels of cholesterol, 24OHC and 27OHC and their effects on APP processing are also depicted. *Adapted from Bjorkhem and Meaney 2004 and Chow et al. 2009.*

Several studies have been carried out to understand how altered cholesterol metabolism may affect APP processing. Changes in the subcellular distribution of cholesterol have been found to modulate APP processing, both *in vitro* and *in vivo* (reviewed in Vetrivel & Thinakaran, 2010). Indeed, Bodovitz and Klein (1996) have shown that increasing cholesterol impeded membrane fluidity and lowered soluble APP production, by hampering the protease interaction with its substrate. It was demonstrated, using different means to deliver cholesterol to cultured cells, that increasing cellular cholesterol impairs the association of APP with the membrane, which is known to be a prerequisite for α- secretase activity (Sisodia 1992), thereby dramatically inhibiting this activity (Bodovitz & Klein 1996).

Furthermore, two independent groups have demonstrated that depletion of cholesterol and disruption of lipid rafts affects the amyloidogenic processing of APP, while favoring the non-amyloidogenic pathway (Simons *et al.* 1998, Kojro *et al.* 2001). On the other

hand, Kalvodova and co-workers have demonstrated that, not only β- secretase is located at the lipid rafts, but cholesterol increases its activity as well (Kalvodova *et al.* 2005).

Grimm and co-workers have also shown that β- secretase activity is modulated by cholesterol levels and showed for the first time that γ- secretase activity, which can only cleave APP after α- or β- secretase cleavage, is also affected by cholesterol levels. These authors demonstrated that cholesterol depletion results in a parallel and additive inhibition of both secretases, although the inhibition mechanism seem to be different (Grimm *et al.* 2008).

1.1.5.2 ApoE and AD

As above mentioned, apoE is a key cholesterol transporter that is expressed in the brain and plays an important part in the redistribution of lipids that follows neurodegeneration (Poirier *et al.* 1993, Strittmatter *et al.* 1993). The *APOE* gene presents three allelic variants: ε2, ε3 and ε4. The *APOE* ε3 is the most common allele in the general population, followed by *APOE* ε4 (Strittmatter & Roses 1995). The latter has a dose effect on the distribution of the age of onset in familial AD and has been identified has a major risk factor for both the familial and the sporadic form of AD (Corder *et al.* 1993). Each *APOE* ε4 allele inherited lowers the distribution of the age of onset and increases the risk of developing the disease (Corder *et al.* 1993). In contrast, it has been shown that the inheritance of an *APOE* ε2 allele decreases the risk and increases the mean age of onset of AD (Corder *et al.* 1994).

ApoE co-localizes with senile plaques (Namba *et al.* 1991) and Aβ levels are increased in the brains of AD patients carriers of the *APOE* ε4 allele (Schmechel *et al.* 1993). Furthermore, it has been shown that apoE is capable of reducing potentially toxic extracellular Aβ (Beffert *et al.* 1999). It has been demonstrated that both *APOE* ε2 and ε3, but not ε4, play important roles in the clearance of Aβ from the extracellular space via the low-density lipoprotein receptor-related protein (LRP) (Yang *et al.* 1999).

Other authors have also shown that apoE4 appears to modulate APP processing and Aβ production through the LRP pathway. By stably transfecting cells with human APP, Ye and co-workers (2005), showed that both apoE3 and apoE4 increase Aβ production. However, they have also demonstrated that the stimulatory effect of apoE4 on Aβ production was linked to an intramolecular interaction between two structural domains of

apoE4, which does not happen in the apoE3. Therefore, there is an isoform-specific effect of apoE4 on the LRP pathway-dependent increase of Aβ production (Ye *et al.* 2005).

A more recent study demonstrates a cross-talk between APP processing, apoE expression and cholesterol metabolism (Liu *et al.* 2007). Using conditional knockout mice, it was demonstrated that AICD, resulting from the two pathways of APP cleavage, can down-regulate LRP1 expression, which in turn modulates apoE and cholesterol levels within the CNS. In fact, not only cholesterol levels seem to regulate APP processing, but also AICD, a proteolytic fragment of APP, can alter the redistribution of cholesterol in the CNS.

1.1.5.3 Statins in AD

As mentioned above, statins are cholesterol lowering drugs that inhibit HMG-CoA reductase. It is known that different statins can traverse the BBB in varying degrees, giving rise to pleiotropic effects, namely decreasing the Aβ load, although the mechanism by which these compounds act is not currently well known (Vega *et al.* 2003).

The Hartmann laboratory was the first to try to dissect the mechanisms underlying the effect of statins on AD. It was shown that simvastatin can have a beneficial effect in AD, since this cholesterol-lowering drug and a cholesterol-extracting toxin strongly reduced neuronal Aβ levels *in vitro*. Moreover, guinea pigs treated with simvastatin also had reduced levels of cerebral Aβ (Fassbender *et al.* 2001). Several other studies sustain the idea that statins may have a beneficial effect in AD by affecting the cholesterol content, both intracellularly and at the membrane level (Kirsch *et al.* 2003, Urano *et al.* 2005, Burns *et al.* 2006), which could in turn affect APP processing. On the other hand, several other studies support the hypothesis that statins have a positive effect in AD, due to their anti-inflammatory properties (Cordle & Landreth 2005, Chauhan *et al.* 2004, Tong *et al.* 2009). Moreover, Sun and co-workers have shown that pre-treatment of human glioma cells with pravastatin decreases Aβ dependent pro-inflammatory markers (Sun *et al.* 2003).

Isoprenoid intermediates of the mevalonate pathway are important for prenylation of small guanosine triphosphate binding proteins, which regulate vesicular transport and cytoskeletal membrane interactions (Sinensky 2000). It has been postulated that one or more of these intermediates are crucial for long-term potentiation (LTP), at least in a subset of neurons (Kotti *et al.* 2006). Several recent studies have shown that statins, by

altering the levels of isoprenoid intermediates, affect neurite outgrowth (Pooler *et al.* 2006), inhibit vesicular trafficking and impair Aβ secretion (Ostrowski *et al.* 2007).

The beneficial effect of statins in AD still remains controversial as several epidemiological studies show that the use of statins is correlated with amelioration of AD symptoms (Fassbender *et al.* 2002, Sjogren *et al.* 2003, Hoglund *et al.* 2005), while others fail to show any improvement (Tokuda *et al.* 2001, Ishii *et al.* 2003, Hoglund *et al.* 2004).

1.1.5.4 Oxysterols and AD

Several studies have described changes in the levels of 24OHC and 27OHC in AD. Indeed, the plasma levels of 24OHC were shown to be significantly lower in the brain of AD patients when compared with controls, whereas the levels of 27OHC were increased (Heverin *et al.* 2004).

Based on a comprehensive examination of total cholesterol, cholesterol precursors and oxysterols from brain samples of a large number of subjects, Hascalovici and co-workers (2009) have provided direct evidence of cholesterol homeostasis disruption in the brains of AD and mild cognitive impairment patients. Among other changes, these authors have shown that AD subjects exhibited a decrease in 24OHC with age, relative to subjects with no cognitive impairment and propose that the observed decrease can be the result of an increase in free cholesterol, due to the destruction of myelin sheaths and cell membranes, which exceeds the brain's ability to eliminate cholesterol by conversion into 24OHC (Hascalovici *et al.* 2009).

Brown III and co-workers were the first to demonstrate that 24OHC inhibited the formation of Aβ (Brown *et al.* 2004). Later, the activities involved in this effect were determined (Figure 1.3): it was shown that 24OHC stimulates α- secretase activity, inhibits β- secretase activity and also significantly increases the α-/ β- secretase activity ratio in neuronal cells (Famer *et al.* 2007). Prasanthi and co-workers not only corroborated these results, but they also demonstrated that 27OHC increased β- secretase activity and suggested that the 24OHC/ 27OHC ratio could be important in APP processing (Prasanthi *et al.* 2009). These results together with the fact that there is a flux of 27OHC from the plasma to the brain (Heverin *et al.* 2005, Meaney *et al.* 2007), have lead to the proposal that 27OHC can be the missing link between high plasma cholesterol and AD (Bjorkhem *et al.* 2006).

1.2. CYP46A1

The Cytochrome P450 (CYP) mono-oxigenase constitutes a superfamily of enzymes, responsible for the oxidative, peroxidative and reductive metabolism of a wide range of endogenous compounds such as steroids, bile acids, fatty acids, prostaglandins, leukotrienes, biogenic amines, retinoids, lipid hydroperoxides and phytoalexins (reviewed in Coon 2003). Moreover, these enzymes catalyze the metabolic conversion of a striking number of foreign chemicals, such as drugs, environmental chemicals and pollutants, to polar derivatives prone to be eliminated from the organism (reviewed in Waxman 1999). Although, the metabolism of the exogenous compounds primarily results in detoxification, the action of CYPs can also lead to the formation of toxic metabolites that can contribute to cancer, birth defects and other toxic effects (reviewed in Nebert & Russell 2002).

CYP enzymes are classified into families and subfamilies according to their amino-acid sequence similarities (> 40% and > 55% sequence identity, respectively). The family is designated by an Arabic number, followed by a letter indicating the subfamily identity and an Arabic number indicating the individual enzyme. For human and all other species the gene nomenclature is an italicized root symbol "*CYP*" ("*Cyp*" for mouse and *Drosophila*). The cDNA, mRNA or enzyme are referred to in regular capital letters for all species, without italics or hyphens (Nelson *et al.* 1996).

In humans, 57 functional CYPs have been described (Nelson 2003). CYPs from families 1 to 4 exhibit a broad, but overlapping, substrate and product specificity. These enzymes are primarily responsible for the metabolism of xenobiotics, while also being capable of metabolizing endogenous compounds. The CYPs belonging to the other families play a key role in biosynthetic pathways, being mainly involved in endogenous compounds metabolism. These enzymes participate in thromboxane and prostacyclin synthesis (CYP 5 and 8A), mediate steroid-hormone biosynthesis (CYP 11, 17, 19, and 21), catalyse reactions in the biosynthetic pathways of bile acids, vitamin D3 and cholesterol (CYP 7, 8B, 24, 27, 46 and 51) and, particularly, CYP26 is involved in retinoid metabolism (Waxman 1999, Lund *et al.* 1999).

The expression of different CYPs can be development- and tissue-specific, regulated by endogenous hormones and cytokines and/ or respond to structurally diverse exogenous compounds, which often increase P450 protein levels by stimulating P450 gene transcription initiation (reviewed in Waxman 1999). Furthermore, CYP expression is

often induced by accumulation of the substrate (Nebert & Russell 2002). Taken together, these findings make cytochromes P450 genes as an attractive model for the study of gene regulation.

The *CYP46A1* gene codes for a highly conserved cholesterol 24-hydroxylase, that belongs to the CYP superfamily. This enzyme is responsible for the hydroxylation of cholesterol, by introducing a hydroxyl group at carbon 24 of this molecule (Russell *et al.* 2009). The human *CYP46A1* was found to be located at chromosome 14q32.1. Both the human and mouse genes were sequenced and each contain 15 exons and 14 introns, with all introns interrupting the human and mouse genes at the same positions. CYP46A1 from both species are microsomal proteins that comprise 500 amino-acids, and share a degree of sequence identity of 95% (Lund *et al.* 1999).

This cytochrome P450 is predominately expressed in the CNS, unlike other P450 sterol-hydroxylases that are predominantly expressed in the liver (Xie *et al.* 2003). Of the 16 human tissues screened by Lund and co-workers, the CYP46A1 mRNA was only detectable in the brain. *CYP46A1* is broadly expressed throughout the brain and its mRNA is detected at higher levels in the gray matter, such as the putamen, cerebral cortex and caudate nucleus and at lower levels in the white matter (Lund *et al.* 1999). More recently, it was shown that the cholesterol 24-hydroxylase is also expressed in neurons of the neural retina (Bretillon *et al.* 2007).

A more detailed analysis was performed in the mouse brain, in which both immunohistochemistry and mRNA *in situ* hybridization revealed that CYP46A1 is expressed in all the six layers of the cerebral cortex, the hippocampus, dentate gyrus and thalamus, but not in support cells or the white matter of the adult brain (Lund *et al.* 1999). At the sub-cellular level, this microsomal cytochrome P450 is localized at the endoplasmic reticulum. Furthermore, this enzyme was shown to be distributed throughout the cell bodies and dendrites of several types of neurons of the mouse brain (Ramirez *et al.* 2008).

The mouse CYP46A1 mRNA and protein are both expressed in the brain at birth, and 24OHC is detected in the serum one day after birth. The serum levels of this oxysterol peak between post-natal days 12 and 15 during the time of myelination (Lund *et al.* 2003). In accordance, in humans at the age of approximately 1 year, expression levels of cholesterol 24-hydroxylase reach a steady state that is maintained during adulthood. The increase in production and secretion of 24OHC during myelination may reflect a dual role for this oxysterol both as a vehicle for reverse cholesterol transport and as a mediator of

intercellular signaling between neurons and support cells (Lund *et al.* 1999). As previously stated 24OHC is a ligand for the LXR (Lehmann *et al.* 1997), which regulate a number of genes involved in fatty acid metabolism (Chawla *et al.* 2001). Moreover, LXR α/β null mice show a variety of CNS defects upon aging including lipid accumulation, astrocyte proliferation, and disorganized myelin sheaths (Wang *et al.* 2002). Furthermore, CYP46A1 has also been proposed to be part of a system for the removal of damaged cell membranes after traumatic brain injury, by the conversion of cholesterol to 24OHC, but also by activation of LXR-regulated gene transcription (Cartagena *et al.* 2008).

1.2.1. Protein function

After cloning of the CYP46A1 cDNA (Lund *et al.* 1999), it was possible to characterize this enzyme at the biochemical level. Studies from Irina Pikuleva's laboratory have demonstrated that CYP46A1 has a very broad subtract specificity and that besides metabolizing cholesterol it is able to metabolize several endogenous and exogenous compounds (Mast *et al.* 2003). Firstly, by transfecting human embryonic kidney 293 cells with a CYP46A1 cDNA expression vector, the authors demonstrated that CYP46A1 had affinity towards cholesterol but also indicated that 24-hydroxylation of the cholesterol molecule was not the only activity of this enzyme. To further characterize the CYP46A1 activities, the same authors developed an *E. coli* expression system and partially purified the recombinant enzyme. This lead to the discovery that CYP46A1, not only metabolizes cholesterol into 24OHC but it also further metabolizes this oxysterol originating 24,25- and 24,27- dihydroxycholesterols. It was demonstrated that the enzyme has 80-fold more affinity for 24OHC than for cholesterol, with the oxysterol being hydroxylated 30 times faster than cholesterol. Moreover, CYP46A1 was found to hydroxylate diverse endogenous steroids, such as 7α-hydroxycholesterol, cholestanol, cortisol, testosterone and progesterone (Mast *et al.* 2003). This activity towards endogenous steroids may have physiological significance since the brain is a steroidogenic organ, in which steroid hormones are synthesized either *de novo* from cholesterol or from blood-born steroidal precursors. However, the possible involvement of CYP46A1 in the neurosteroidogenesis and degradation pathways and are not completely understood.

Several xenobiotics are also metabolized by this enzyme, namely bufuralol an adrenoceptor antagonist; dextromethorphan an antitussive drug; diclofenac a non-

steroidal anti-inflammatory drug; and phenacetin an analgesic drug; all of which are structurally diverse compounds, pointing to a wide range of substrate specificity (Mast *et al.* 2003). In order to further elucidated the physiological importance of CYP46A1 in xenobiotic metabolism, the same authors have engineered the CYP46A1, truncating the N-terminal amino-acid residues, and making the enzyme more soluble (Mast *et al.* 2004). Using this engineered protein, it was shown that structurally distinct compounds can bind to the enzyme active site and identified several strong inhibitors and mild activators, hypothesizing that CYP46A1 activity could be altered by exposure to some therapeutic drugs and potentially other xenobiotics (Mast *et al.* 2008). In fact, the effect of voriconazole, an antifungal drug, was further assessed. This drug was shown to inhibit CYP46A1 activity. Both *in vitro*, and *in vivo* studies, have demonstrated that this drug not only passes the BBB, but also decreases 24OHC and other intermediaries of the cholesterol biosynthetic pathway (Shafaati *et al.* 2010). The same authors have also demonstrated that dietary intake of n-3 polyunsaturated fatty acids increase the CYP46A1 mRNA levels in the brains of hamsters, although they failed to demonstrate an increase in the activity of the enzyme or in 24OHC levels (Mast *et al.* 2010).

To further understand the role of this key enzyme in cholesterol turnover in the brain, knockout mice for *Cyp46a1* were produced by David Russell's group. These mice look outwardly normal and have no differences in the growth rates and body weight when compared to the wild-type. However, *Cyp46a1*$^{-/-}$ mice have an approximately 65% reduction in brain cholesterol excretion. This decrease is compensated for by a reduction of approximately 60% in the *de novo* synthesis (Figure 1.4), in brain regions that normally express high levels of CYP46A1, whereas in parts of the brain where CYP46A1 is expressed at low levels there is a reduction of the synthesis of about 20-30%, such as the steady-state levels of cholesterol remain constant (Lund *et al.* 2003, Russell *et al.* 2009). Whole body cholesterol synthesis was not significantly different between the wild-type and the knockout mice (Lund *et al.* 2003).

Further characterization using learning tests and electrophysiological studies, showed that *Cyp46a1* null mice exhibit severe deficiencies in spatial, associative and motor learning and hippocampal LTP. The incubation of wild-type hippocampal slices with compactin, a HMG-CoA reductase inhibitor, also impaired LTP. Furthermore, LTP was restored by the addition of geranyl-geraniol, an isoprenoid intermediate that cannot be converted to cholesterol (Kotti *et al.* 2006). These data point to an important role of

cholesterol turnover in the production of isoprenoid intermediates that are essential in LTP and learning.

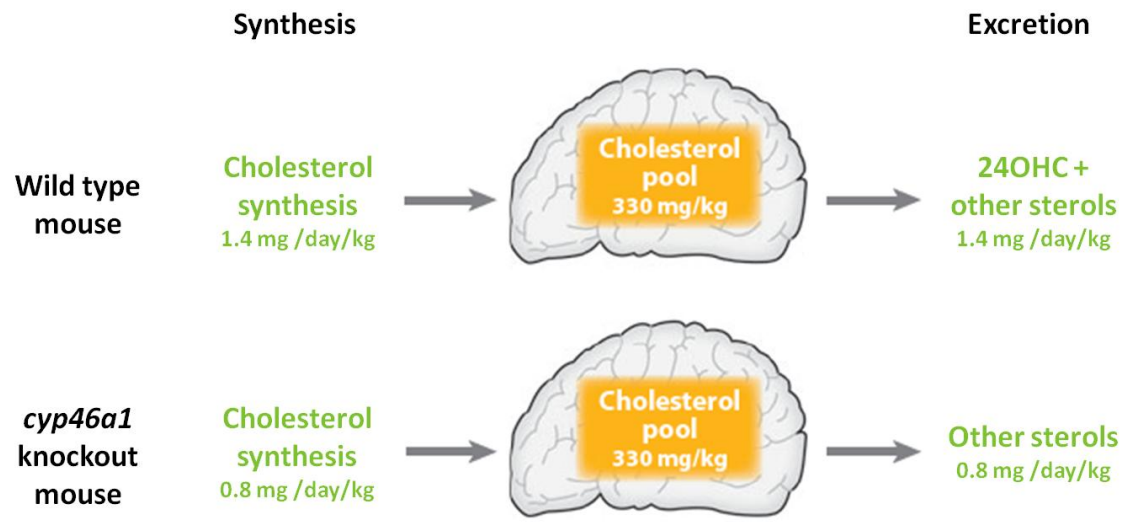

Figure 1.4. Cholesterol turnover in wild-type and *Cyp46a1* knockout mouse. Knockout mice lacking *Cyp46a1* have an approximately 65% reduction in brain cholesterol excretion, but this decrease is compensated for by a reduction in the *de novo* synthesis, so that the steady-state levels of cholesterol remain constant. *Adapted from Russell et al. 2009.*

1.2.3. CYP46A1 and AD

In the last years, several epidemiological studies have reported that genetic variants of *CYP46A1* might act as risk factor for AD. Different intronic polymorphisms in *CYP46A1* have been described to influence AD risk and cholesterol levels or Aβ load (Kolsch *et al.* 2002, Papassotiropoulos *et al.* 2003, Combarros *et al.* 2004, Borroni *et al.* 2004, Wang *et al.* 2004, Golanska *et al.* 2005, Helisalmi *et al.* 2006, Fernandez Del Pozo *et al.* 2006, Li *et al.* 2006, Ma *et al.* 2006, Kolsch *et al.* 2009). These results are nonetheless controversial, since in other studies, no such association was found (Desai *et al.* 2002, Johansson *et al.* 2004, Chalmers *et al.* 2004, Kabbara *et al.* 2004, Ingelsson *et al.* 2004, Juhasz *et al.* 2005, Shibata *et al.* 2006, Tedde *et al.* 2006, Wang & Jia 2007, Llorca *et al.* 2008, Golanska *et al.* 2009). A list of all the published association studies for *CYP46A1* genetic variants and AD is summarized in Table 1.1.

Table 1.1. Overview of published association studies for *CYP46A1* genetic variants and AD

SNP	Association	Population	Reference
rs754203 and rs4900442	Positive	Germany	(Kolsch *et al.* 2002)
rs754203	Negative	USA	(Desai *et al.* 2002)
rs754203	Positive	Switzerland, Greece and Italy	(Papassotiropoulos *et al.* 2003)
rs754203 and rs4900442	Negative	Sweden and Scotland	(Johansson *et al.* 2004)
rs754203	Positive	Spain	(Combarros *et al.* 2004)
rs754203	Negative	United Kingdom	(Chalmers *et al.* 2004)
rs754203	Positive	Italy	(Borroni *et al.* 2004)
rs754203	Negative	France	(Kabbara *et al.* 2004)
rs754203	Positive	China	(Wang *et al.* 2004)
rs754203	Negative	USA	(Ingelsson *et al.* 2004)
rs754203	Positive	Poland	(Golanska *et al.* 2005)
rs754203	Negative	Hungary	(Juhasz *et al.* 2005)
rs754203	Positive	Finland	(Helisalmi *et al.* 2006)
rs754203 and rs4900442	Negative	USA and Canada	(Shibata *et al.* 2006)
rs754203	Positive	Spain	(Fernandez Del Pozo *et al.* 2006)
rs754203	Negative	Italy	(Tedde *et al.* 2006)
rs754203 and rs4900442	Positive	China	(Li *et al.* 2006)
rs754203, rs3742375, rs3742376 and rs3742377	Positive	China	(Ma *et al.* 2006)
rs754203	Negative	China	(Wang & Jia 2007)
rs754203 and rs4900442	Negative	Mixed	(Llorca *et al.* 2008)
16 different SNPs	Positive (rs754203 and rs4900442)	Germany	(Kolsch *et al.* 2009)
rs754203	Negative	Sweden	(Golanska *et al.* 2009)

Adapted from www.alzforum.org.

Moreover, the functional significance of these polymorphisms is not known, since none of the studied polymorphisms is located in *CYP46A1* exonic regions, and only 4 of the polymorphisms studied by Llorca and co-workers are predicted to be near an exon/ intron boundary (Llorca *et al.* 2008).

In a recent study, two promoter polymorphisms were identified and associated with AD in a Chinese population. These two polymorphisms could putatively impair the binding of several transcription factors to the *CYP46A1* proximal promoter, since directed mutagenesis analysis revealed a decrease in reporter promoter activity (Li *et al.* 2010). Nevertheless, only *in silico* predictions of the potential transcriptional regulators were made, and the functionality of the putative binding sites should be further assessed.

Although the molecular epidemiology studies do not reveal a clear association of particular *CYP46A1* polymorphisms with AD incidence, other significant evidences suggest that CYP46A1 could affect the pathophysiology of AD. *In vitro* studies have shown that overexpression of CYP46A1 and/ or treatment with 24OHC can reduce membrane cholesterol levels, that can lead to the dissociation of several proteins from lipid rafts impairing their function, and can increase α-secretase activity as well as α/ β-secretase activity ratio, by further inhibiting β- secretase activity (Famer *et al.* 2007, Prasanthi *et al.* 2009, Hudry *et al.* 2009, Tian *et al.* 2010).

Hudry *et al.* (2009) published a study where two different transgenic mouse strains that develop Aβ plaques and memory deficits with different timings were used: APP23 mice that develop memory deficits at 3 months and amyloid plaques at 6 months; and APP/ PS1 mice that develop amyloid plaques at 10 weeks and are characterized by a more severe phenotype. These authors have showed that overexpression of CYP46A1 in hippocampal and cortical neurons with an adeno-associated expression vector improved spatial memory before the onset of the Aβ plaques and reduced APP amyloidogenic processing before and after Aβ plaque onset. Furthermore, they have shown that cholesterol 24-hydroxylase overexpression led to a decrease in cholesterol content at the level of lipid rafts and a decreased in APP and components of the γ -secretase in these microdomains (Hudry *et al.* 2009).

In a related study, the ablation of ACAT-1 in triple transgenic mice of AD (3xTgAD) ameliorated cognitive deficits (Bryleva *et al.* 2010). Furthermore, lack of ACAT-1 activity increased the content of 24OHC in the brain of these animals, decreased cholesterol synthesis and lead to more than 60% reduction in full-length human APP as well as its proteolytic fragments. Moreover, treatment of primary neurons with 24OHC caused a rapid decline in APP and in HMG-CoA reductase protein levels. The authors propose an explanatory model of the cross-talk between ACAT-1, cholesterol metabolism and APP processing (Figure 1.5) (Bryleva *et al.* 2010).

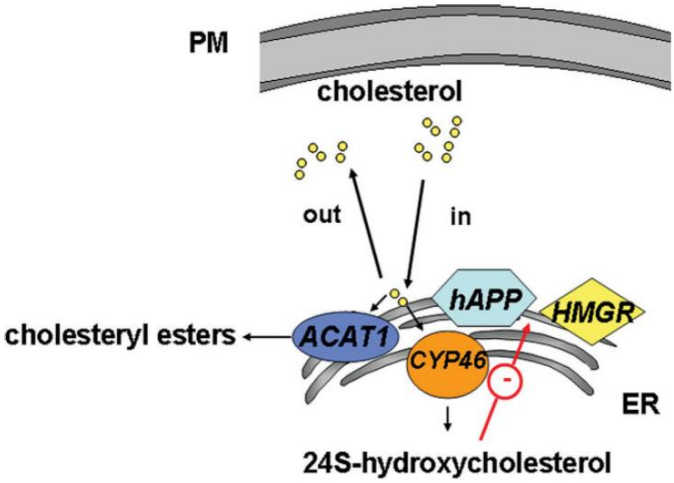

Figure 1.5. Proposed model linking ACAT1, cholesterol and APP processing. Inhibition of ACAT1 increases free cholesterol that is converted by CYP46A1 into 24OHC. The increased content in 24OHC and cholesterol inhibit APP levels and processing, and HMG-CoA reductase activity at the endoplasmic reticulum. *Adapted from Bryleva et al.(2010).*

It was also shown that up-regulation of *CYP46A1* leads to the activation of tyrosine kinase (Trk) B receptor in immature neurons in the absence of its cognate ligand - BDNF (Martin *et al.* 2008). This auto-activation is possibly triggered by an alteration in the cholesterol content in membrane lipid rafts and leads to the activation of survival pathways. In subsequent studies the same authors have shown that knockdown of *CYP46A1* in hipoccampal neurons leads to a significant increase in apoptosis (Martin *et al.* 2009). Interestingly, TrkB receptor activation by BDNF modulates the expression of several genes of the cholesterol biosynthetic pathway in neurons but not in glial cells (Suzuki *et al.* 2007).

Although the latter studies suggest that induction of *CYP46A1* can have a therapeutic value in the management of AD progression, this upregulation seems to be beneficial only if specifically targeted to neuronal cells. Indeed, it has been shown that overexpression of CYP46A1 in primary cultures of astrocytes decreases the cholesterol content of lipid rafts, and consequently results in a dissociation of the excitatory amino-acid transporter 2 (EAAT2) from these microdomains, and the loss of EAAT2 and associated glutamate uptake function, which is deleterious to neuronal cells (Tian *et al.* 2010).

Furthermore, a transgenic *Cyp46a1*$^{-/-}$/AD mouse strain was developed by crossing the *Cyp46a1* knockout mouse strain with a AD transgenic mouse strain [B6.Cg-Tg(APPswe, PSEN1E9)85Dbo/J] that accumulates Aβ plaques in the cortex at an early age and present cognitive defects (Halford & Russell 2009). These authors have shown that decreasing

cholesterol *de novo* synthesis in the brain does not markedly influence the accumulation of Aβ plaques in these animals. Interestingly, it was also demonstrated that the *Cyp46a1*$^{-/-}$ / AD animals have a prolonged life-span when compared to the WT/AD mice, suggesting that reduction of *de novo* brain cholesterol synthesis does not seem to alter the progress of AD, however loss of one or both the *Cyp46a1* alleles may confer a survival advantage (Halford & Russell 2009).

These studies underscore the importance of CYP46A1 in the maintenance of cellular cholesterol levels and its importance in the activation/ repression of several important proteins that are related to different cell processes.

Independently of its putative role on the onset/ progression of AD, *CYP46A1* expression profile is altered in *post-mortem* brains of AD patients when compared with the controls. Indeed, CYP46A1 was shown to be up-regulated in glial cells in the brains of AD patients. Oligodendrocytes showed very weak staining, whereas some astrocytes stained positive for CYP46A1 preferentially in the soma and partially in the fibrillar branching, whereas neuronal cells show a decrease in CYP46A1 (Bogdanovic *et al.* 2001). These results were corroborated by another group that demonstrated that in the brains of AD patients CYP46A1 shows prominent expression in astrocytes and in degenerating neurites around senile plaques, and neurons have a less marked expression of CYP46A1 in the brains of AD patients, when compared to controls (Brown *et al.* 2004).

Moreover, several studies have demonstrated an upregulation of CYP46A1 in response to brain injury. After brain injury induced by the excitotoxin, kainic acid, He and co-workers were the first to demonstrate increased CYP46A1 expression in rat astrocytes (He *et al.* 2006). In this study, the levels of both cholesterol and 24OHC were shown to be increased after injury, due to an increase in cholesterol synthesis and the upregulation of CYP46A1 in the degenerating brain tissue, and not by an increase in plasma uptake (He *et al.* 2006).

In a rat model of multiple sclerosis with a compromised BBB, the observed increased expression of CYP46A1 in infiltrated macrophages near a site of injury was proposed to facilitate metabolism of phagocytosed cholesterol from neural membranes and myelin. The presence of CYP46A1 in macrophages near blood vessels may allow for rapid excretion of cholesterol into the circulation (Teunissen *et al.* 2007). In addition, activated microglia cells expressing CYP46A1 were detected at the site of injury in a rat model of traumatic brain injury (Cartagena *et al.* 2008). These results were corroborated in a more

recent study, where it was demonstrated that microglial cells surrounding lesion sites have increased CYP46A1 expression (Smiljanic *et al.* 2010).

These studies give emphasis to the importance of CYP46A1 in maintaining brain cholesterol homeostasis in response to CNS injury, by facilitating the elimination of excess cholesterol released from damaged neuronal membranes and myelin phagocytosed by activated microglia and astrocytes.

1.2.4. Gene regulation

Ingemar Björkhem's group was the first to structurally and functionally characterize the human *CYP46A1* promoter (Ohyama *et al.* 2006). These authors cloned 2.7 Kb of the *CYP46A1* 5´ flanking region and found that it lacks TATA or CAAT boxes. The proximal promoter is very GC rich, a feature often found in genes considered to have a largely housekeeping function.

The functional analysis of the *CYP46A1* promoter indicated that the region between nucleotides -659 and -510 was responsible for basal promoter activity and most likely silencing elements were present further upstream. Nevertheless, these authors could not identify in this region putative binding sites for neuron-specific transcription factors. Furthermore, they demonstrated that there is no substrate-dependent transcriptional regulation and that treatment of neuroblastoma cells with a broad spectrum of endogenous and exogenous compounds did not result in any significant change in *CYP46A1* promoter activity. However, treatment with a radical generator slightly increased *CYP46A1* reporter activity, pointing to a possible role of oxidative stress in the regulation of *CYP46A1* expression (Ohyama *et al.* 2006).

In a follow-up study it was demonstrated that epigenetic mechanisms might be involved in the tissue-specific expression of *CYP46A1*. Treatment of neuroblastoma cells with trichostatin A (TSA) a potent inhibitor of histone deacetylases (HDAC) led to a marked time-dependent derepression of *CYP46A1* expression. The effect of HDAC inhibitors was also evaluated *in vivo* and corroborated the *in vitro* findings of a dose dependent increase in the expression of *CYP46A1* in the brain (Shafaati *et al.* 2009). Our group has also corroborated the findings by Shafaati *et al.* (2009) showing that inhibition of HDAC activity by TSA, valproic acid and sodium butyrate caused a potent induction of *CYP46A1* expression (Nunes *et al.* 2010). Furthermore, our studies have shown that silencing the Specificity protein (Sp) transcription factors led to a significant decrease in

HDAC inhibitor-mediated induction. Moreover, our data showed that a decrease in Sp3 binding at particular responsive elements, can shift the Sp1/Sp3/Sp4 ratio, and favor the detachment of HDAC 1 and 2 and the recruitment of transcriptional activators (Nunes *et al.* 2010). These data imply that an epigenetic program may be responsible for the tissue specific expression of *CYP46A1*.

1.3 Transcriptional regulation of gene expression

Eukaryotic organisms, especially mammals, have evolved complex gene regulatory mechanisms that control tissue- and development- specific gene expression. Gene transcription is a remarkably intricate process that is tightly regulated by the interplay between structural properties of DNA, chromatin dynamics, and transcription factors (TF) that target specific DNA sequences, which interact with components of the core initiation machinery leading to the activation or repression of transcription (Kadonaga 2004).

In fact, genes contain complex arrays of specific DNA sequences such as promoters, enhancers, silencers and insulators that are positioned throughout the genome and that cooperate with epigenetic marks to define specific expression patterns (Figure 1.6) (Lemon & Tjian 2000).

The core promoter plays a pivotal role in the transcription process and is defined as the DNA region that directs the accurate initiation of transcription by RNA polymerase II (RNA pol II) (Juven-Gershon & Kadonaga 2010). The conserved TATA box was the first eukaryotic core promoter element identified and besides the TATA box-enriched promoters, a second class of promoters exists, the CpG-rich promoters (Carninci *et al.* 2006). Although these CpG island-associated promoters are predominant throughout the genome, the mechanisms and factors responsible for their function remain poorly identified (Baumann *et al.* 2010).

In eukaryotes, the core promoter is recognized by the general transcription factor complexes, such as TFIID, which consists of the TATA binding protein (TBP) and approximately 13 or 14 TBP-associated Factors (TAFs). Multiple TFIID related complexes exist and function at distinct promoters through the use of tissue-specific TAFs and TBP-related factors (Levine & Tjian 2003). Moreover, it is known that these complexes function in the majority, if not all, promoters recognized by RNA pol II, regardless of the presence or absence of the TATA box (Goodrich & Tjian 2010).

The DNA motifs present at the promoter, silencers and enhancers are recognized by sequence-specific DNA binding regulatory proteins, which bind directly to DNA and, through protein–protein interactions, convey signals to the basal transcriptional machinery resulting in differential gene expression. These DNA binding proteins can recruit a number of different cofactor complexes, which can be co-activators or co-repressors that in turn can serve as bridges between distal regulatory sequences and the core promoter or can induce conformational modifications important for regulating transcription (Levine & Tjian 2003).

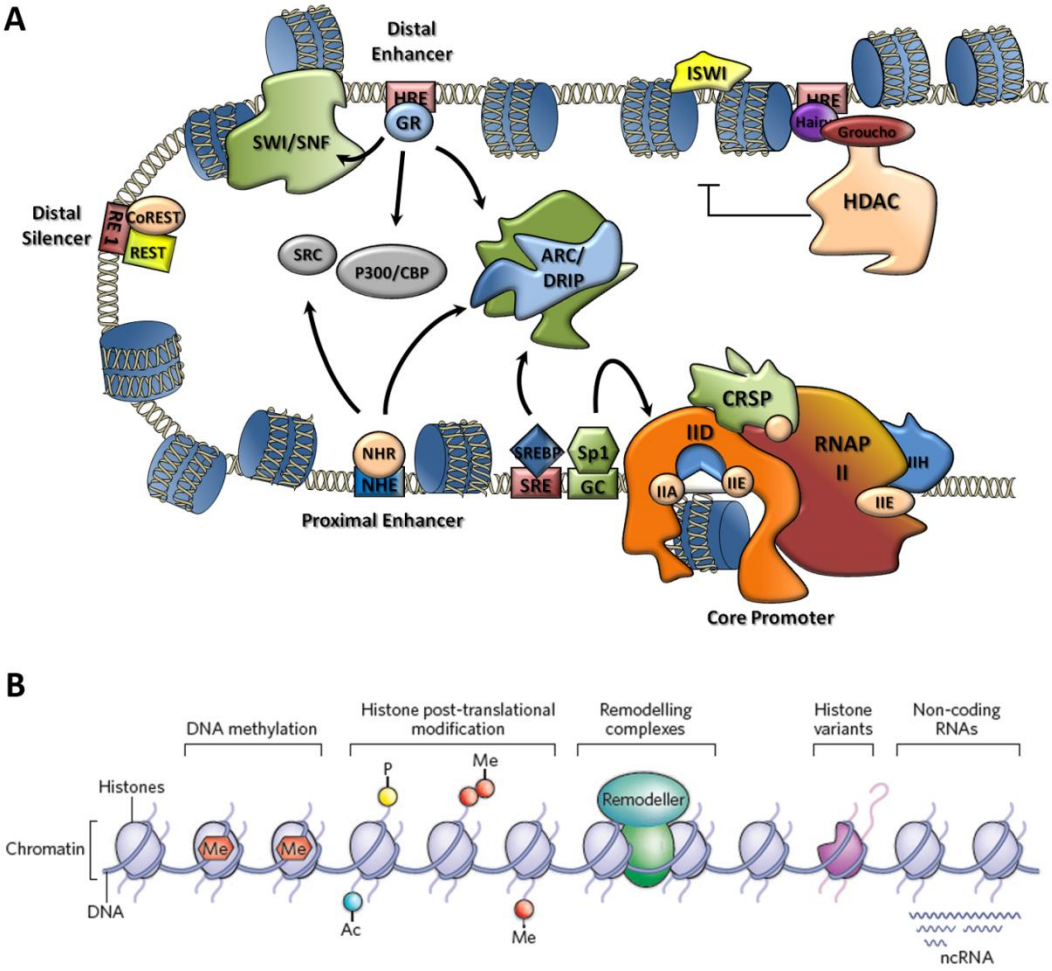

Figure 1.6. Fundamental elements of eukaryotic transcriptional control. A) Scheme representing the essential components involved in gene regulation, including promoter, silencer and enhancer DNA sequences, sequence-specific DNA-binding transcription factors (i.e. GR, NHR, Hairy, SREBP, Sp1, REST), components of the core initiation machinery (TFIIA, IIB, IID, IIE, IIF, IIH) required by RNA polimerase II for promoter recognition and basal transcription, co-activators (p300/CBP, ARC/DRIP, CRSP, SAGA), co-repressors (Groucho, CRSP) and chromatin modifying complexes (HDAC, SWI/SNF, ISWI). B) Scheme representing the epigenetic modifications involved in the control of gene expression: DNA methylation, histone modifications, chromatin remodeling complexes, histone variants, and non-coding RNAs (ncRNAs). *Adapted from Lemon & Tjian 2000 and Dulac 2010.*

Epigenetic mechanisms are also important for gene regulation. DNA methylation is mainly confined to CpG dinucleotides in mammals. The methylation of cytosine nucleotides in DNA to form 5-methylcytosine has an important role in the silencing of individual genes, the constitutive silencing of chromatin regions, the inactivation of one of the X chromosomes in females and the imprinting of parental alleles (Bird 2007).

Gene expression can be further regulated by modifications at the chromatin level. In addition to the structural role for histones, post-translational modifications (PTM) of their N-terminal tails give rise to changes in nucleosome–DNA interactions that are important for transcriptional regulation. In fact, histones can suffer a variety of modifications including methylation, acetylation, ubiquitination, ADP-ribosylation, small ubiquitin-like modifier modification (SUMOylation) and phosphorylation, and these in turn regulate gene expression by allowing differential binding of specific TFs, co-activators, co-repressors and the constituents of the transcriptional machinery (Jenuwein & Allis 2001). Although the significance of most of these modifications is not fully understood, acetylation and methylation of specific lysines have been demonstrated to be key modulator marks for transcriptional activation or repression (Kouzarides 2007).

Additionally, a class of recently discovered RNA molecules, the non-coding RNAs, was found to regulate transcription by epigenetic mechanisms. Small interfering RNAs can specifically target particular DNA sequences and silence gene expression in a process that involves histone modifications and DNA methylation (Yilmaz & Grotewold 2010). Moreover, long non-coding RNAs can regulate gene transcription through a diversity of mechanisms and their functions include roles in high-order chromosomal dynamics, telomere biology and sub-cellular structural organization (Mercer *et al.* 2009).

The development and function of the CNS requires accurate gene transcription control in response to environmental signals. Epigenetic mechanisms, such as DNA methylation, histone modification and small-non coding RNAs, are critically important in controlling precise neural gene regulation. Transcription factors also operate and are activated in a sequential order, in particular during neural development, so that many changes occur in response to transcription factors, DNA methylation, small non-coding RNAs and modifications at the chromatin level (Buckley 2007, Ooi & Wood 2008). The precisely controlled interplay between all these genetic and epigenetic events in response to external cues is what determines accurate gene regulation.

1.3.1. DNA methylation

In vertebrates, DNA methylation mainly occurs at the fifth carbon position of the cytosine residue in a CpG dinucleotide. Nevertheless, the eukaryotic genomes are not methylated uniformly but contain methylated regions interspersed with unmethylated domains. Non-coding regions, both intronic and intergenic are heavily methylated to preserve genome integrity (Wilson *et al.* 2007). DNA methylation at promoter regions is much more complex. Indeed, one of the most striking features of vertebrate DNA methylation patterns is the presence of CpG islands, unmethylated GC-rich regions that possess high relative densities of CpG and are positioned at the 5' ends of many genes (reviewed in Illingworth & Bird 2009). The great majority of CpG islands are unmethylated at all stages of development and in all tissue types (Antequera and Bird 1993) even when their associated gene is silent. A small but significant proportion of all CpG islands become methylated during development and usually the degree of methylation correlates with transcription repression. Inhibition of gene expression by DNA methylation is achieved by either directly interfering with transcription factors binding to DNA or by allowing the recruitment of methyl-CpG binding domain (MBD) proteins, which complex with other chromatin modifying proteins that alter chromatin to a repressive state (Feng *et al.* 2007, Metivier *et al.* 2008).

The enzymatic activity of DNA methyltransferases (DNMTs) is responsible for the methylation of the cytosine residue and S-adenosyl methionine (SAM) is the methyl donor for this reaction (Bird 1986), which is cyclic and depends on the clearance of by-products and the replenishment of substrates. Also essential in this process is the presence of specific co-factors, folate, vitamin B6 and vitamin B12 (Narayan & Dragunow 2010).

De novo DNA methylation patterns are established by members of the DNMT3 family and established methylation patterns are maintained by the DNMT1 methyltransferase (Figure 1.7) (Kim *et al.* 2009a) and tailored by the action of different demethylases, although the chemical process by which these enzymes convert the methylated cytosines to unmethylated cytosines is not well established (Brown *et al.* 2008, Day & Sweatt 2010).

The importance of DNA methylation in gene regulation associated with brain function and neurological disorders was first suggested when the gene encoding Methyl-CpG-Binding Protein 2 (MECP2) was identified as the target of mutations that cause Rett syndrome (Amir *et al.* 1999). Interestingly, it has also been demonstrated that certain

genes undergo rapid and specific methylation during LTP (Levenson *et al.* 2006). Recent studies indicate that DNA methylation is dynamically regulated in the adult CNS and that this epigenetic modification may serve as a contributing mechanism in memory formation and storage (reviewed in Day & Sweatt 2010).

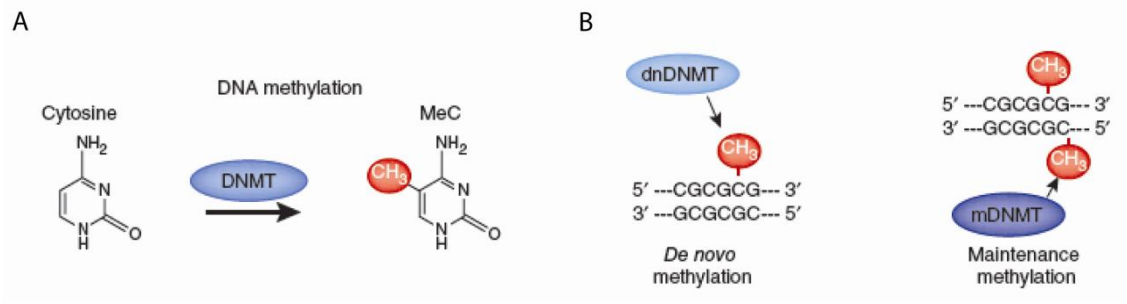

Figure 1.7. DNA methylation. A) DNA methylation occurs at cytosine bases when a methyl group donated by SAM is added at the 5′ position on the pyrimidine ring by a DNMT. B) *De novo* DNMTs methylate previously nonmethylated cytosines, whereas maintenance DNMTs methylate hemi-methylated DNA at the complementary strand. *Adapted from Day & Sweatt 2010.*

Epigenetic mechanisms have been shown to be altered in two degenerative disorders of the CNS – Huntington's disease and AD. Abnormal DNA methylation patterns have been demonstrated to affect AD. The APP promoter region has been shown to be hypomethylated in the brains of AD patients (West *et al.* 1995, Tohgi *et al.* 1999). AD patients have been reported to have elevated levels of homocysteine (Seshadri *et al.* 2002), which can lead to a global hypomehtylation, by the formation of the potent inhibitor of methylation – S-adenosylhomocystein. The genes coding for β- and γ-secretases, key enzymes in the processing of APP, have also been shown to be controlled by DNA methylation (Scarpa *et al.* 2003, Fuso *et al.* 2005). Moreover, inducing demethylation by vitamin B complex deprivation leads to an increase in β- and γ-secretase activity and in the accumulation of Aβ deposition in mice (Fuso *et al.* 2008). Nevertheless, high dose supplements of folate and vitamins B6 and B12 administered to AD patients, induced a decrease in the levels of homocystein, however, this did not reflect in a benefit in cognitive function (Aisen *et al.* 2003, Aisen *et al.* 2008). On contrary, Chan and co-workers employing a supplement cocktail, containing folate, vitamin B6, α-tocopherol, SAM, *N*-acetyl cystein and acetyl-L-carnitine, reported significant improvements in cognitive function in early-stage AD patients (Chan *et al.* 2008).

When studying the cytogenetic effect of the demethylating agent 5-azacytidine in peripheral lymphocyte cultures derived from AD patients and two control groups, it was demonstrated that epigenetic regulation may be compromised in AD patients, since the DNA regions that were methylated before the treatment, were more readily and rapidly unmethylated in AD patients cells (Payao *et al.* 1998). In a recent study with *post-mortem* brain tissue from AD patients it was shown that methylcytosine, DNMT1 and MBD2 were decreased when compared with the controls (Mastroeni *et al.* 2010). All of these studies point to the importance of epigenetic mechanisms in normal brain function and further strengthen the hypothesis of epigenetic dysfunction in AD.

1.3.2 Sequence-specific DNA binding transcription factors

As previously mentioned, in eukaryotes transcription regulation requires precise spatial and temporal cooperation of a multitude of general and sequence-specific transcription factors at *cis*-regulatory elements, including promoters, enhancers, and silencers (Baumann *et al.* 2010).

Sequence-specific DNA binding transcription factors recognize and bind to response elements located at *cis*-regulatory regions, subsequently recruiting TFs and co-factors that act to regulate RNA pol II transcriptional machinery at the core promoter, which in turn mediates the synthesis of RNA transcripts from the DNA template (Kadonaga 2004). The precise regulation of genes does not depend on the binding of a single sequence-specific DNA binding transcription factor, but of multiple factors that can act synergistically and robustly activate transcription in a highly specific manner (Laybourn & Kadonaga 1992).

A significant number of transcription factors are expressed in the brain and are essential for development, differentiation and in regulating gene expression in response to neuronal activity (Ooi & Wood 2008). In fact, it has been shown that the precise timing of neural stem cell differentiation is critically controlled by multiple basic helix-loop-helix (bHLH) TFs, both repressors and activators, and that this control is fundamental for the correct formation of the brain (Kageyama *et al.* 2005). The bHLH repressor-type proteins from the Hes family have been shown to regulate Notch signaling, thus inhibiting neuronal differentiation and promoting maintenance of neural stem cells (Kageyama *et al.* 2005). The bHLH activator-type proteins, which include members of the neurogenic differentiation (NeuroD), neurogenin and Achaete-Scute families have been shown to be involved in neuronal differentiation, by inducing neuronal-specific gene expression but

also by inhibiting glia-specific gene expression (Bertrand *et al.* 2002, Kageyama *et al.* 2005). Furthermore, combinations of distinct bHLH proteins can induce different types of neurons and it has been suggested that distinct bHLH proteins acting in cascades trigger the sequential steps of cell determination and differentiation (Wen *et al.* 2009).

Another important transcription factor that regulates the tissue-specific expression of several neuronal genes, by binding to the repressor element 1 (RE1) present at the promoter of these genes, is the RE1-silencing transcription factor (REST)/ neuron-restrictive silencer factor (NRSF). This transcription factor was found to be a negative regulator rather than a transcriptional silencer of neuronal gene expression, as initially thought, and it is known that it interacts with positive regulators to modulate target gene expression in a quantitative manner in different cell types, including neurons (Ooi & Wood 2007, Palm *et al.* 1998). REST-mediated gene repression is mainly achieved by the recruitment of two separate co-repressor complexes, CoREST and mSin3, and with several complementary chromatin-modifying enzymes, such has HDACs (Huang *et al.* 1999). In addition to high levels of expression in non-neuronal cells, REST is expressed in the brain, where it can repress but does not silence gene expression. In neurons, REST mRNA exists in several alternatively spliced forms, but its function in neurons is not well established (Ooi & Wood 2007).

1.3.2.1 The specificity protein/ Krüppel-like factors transcription factor family

Clearly transcription factors play a pivotal role in regulating gene expression and the specificity protein/ Krüppel-like factors (Sp/ KLF) is one of the best characterized families of transcription factors, especially the prototypical Sp1 (Dynan & Tjian 1983).

This family of transcription factors has several members that share a highly conserved DNA binding domain (DBD) with at least 65% of sequence identity. Structurally, the DBD comprises three adjacent zinc fingers motifs, which allow these transcription factors to bind to GC and GT boxes (5'-GGGGCGGGG-3' and 5'-GGTGTGGGG-3', respectively), with overlapping specificities and affinities (Suske 1999, Suske *et al.* 2005). These motifs are also responsible for protein-protein interaction, which can modulate DNA binding activity of these transcription factors. Furthermore, the protein members of this family contain a nuclear localization sequence immediately adjacent to, or within, the zinc finger sequence (Kaczynski *et al.* 2003).

The Sp/ KLF family is subdivided into two major subfamilies, the Sp family, which has a greater affinity to the GC boxes and share similar N-terminal motifs, and the KLF family, which has greater affinity to the GT boxes (with some exceptions) and is more heterogeneous (Wierstra 2008). These GC and GT boxes are important both in the transcription of ubiquitous as well as tissue-specific genes (Suske 1999). This large family of TFs has at least 25 members, although some of these were discovered only recently and their biological functions are still not known (Suske *et al.* 2005).

The inclusion of TFs to the Sp subfamily is based on their homology and chromosomal localization. Sp1-Sp9 genes are all adjacent to homeobox gene clusters and usually in pairwise combinations (Kawakami *et al.* 2004, Philipsen & Suske 1999, Bouwman & Philipsen 2002). A characteristic feature of these TFs is the presence of a conserved amino-acid stretch named SP box, located close to the N-terminus of the protein, that contains an endoproteolytic cleavage site important for proteasome-dependent degradation (Su *et al.* 1999), and also the presence of the buttonhead (BTD) box, just N-terminal to the zinc-fingers (Suske *et al.* 2005). The BTD box has been shown to be important for the synergistic activation of target genes by Sp1 and SREBP (Athanikar *et al.* 1997).

Within the Sp transcription factors, Sp1, Sp2, Sp3 and Sp4 form a subgroup based on their similar modular structure (Bouwman & Philipsen 2002), having at least one glutamine rich transactivation domain (TAD) critical for transcriptional activation (Figure 1.8). In fact, Sp2 differs from the other Sp proteins of this subgroup, since it only has one TAD and lacks a serine/ threonine rich domain, important for PTMs that regulate the activity of these TFs. In fact, no functional analysis has been made, and little is known about the role of Sp2 (Suske 1999). Sp1, Sp3 and Sp4 all have two glutamine rich TAD (transactivation domains A and B), and all have the serine/ threonine rich region that putatively regulate their activity (Bouwman & Philipsen 2002). The high degree of structural conservation between these three proteins demonstrates that these proteins are all transcriptional regulators that bind to similar DNA elements (Hagen *et al.* 1994).

Sp1 and Sp3 are ubiquitously expressed and necessary for the regulation of many genes that are involved in numerous cellular processes (Cawley *et al.* 2004). In contrast, Sp4 is expressed at very high levels in the adult brain and the retina and also in several other tissues, albeit at lower levels (Hagen *et al.* 1994, Supp *et al.* 1996, Lerner *et al.* 2005).

Sp1 has two isoforms (that differ in the extent of the N-terminus of the proteins) with the longest isoform having 106 kDa and the shortest 96 kDa. Both isoforms contain activation domains at the glutamine rich region, as already referred, however this TF also has an inhibitory domain at the N-terminal end of the protein (Murata *et al.* 1994). The abundance of Spl protein is believed to be invariant in most mammalian cell types and it is possible that levels of active Spl are tightly controlled. The binding of different proteins to the N-terminal end of Sp1 may work by sequestering active Sp1 and impairing its ability to bind to DNA and consequently impede the activation of its target genes transcription (Murata *et al.* 1994). The interaction of Sp1 with a large co-activator complex called co-factor required for Sp1 activation (CRSP) has also been shown to stimulate Sp1-mediated transcription *in vitro* (Ryu *et al.* 1999). Thus, Sp1 can be responsible for transcriptional activation or repression. Furthermore, Sp1 has been shown to be able to synergistically activate transcription by at least two different mechanisms. The synergistic effect can be achieved by Sp1 binding to adjacent Sp-binding sites forming homotypic interactions, through a specific domain (domain D) at the carboxy-terminal domain, leading to the formation of multimeric complexes and not by cooperative binding of Sp1 to adjacent response elements (Pascal & Tjian 1991). An alternative synergistic mechanism requires the interaction of Sp1, via the BTD domain, with SREBP, which may be limited to promoters that naturally contain a single SREBP recognition site (Athanikar *et al.* 1997).

Sp3 has four isoforms, two long forms of about 116 kDa (L1-Sp3 and L2-Sp3) and two short isoforms of approximately 76 kDa (M1-Sp3 and M2-Sp3), which derive from alternative translational start sites at positions 1, 37, 856, and 907. The short isoforms lack the A sub-domain of the TAD and act has repressors or weak activators (Sapetschnig *et al.* 2004). Sp3 has also been shown to be responsible for the activation of certain genes and for the repression of others (reviewed in Li & Davie 2010). Moreover, Sp3 does not have the ability to bind to DNA as multimers or to synergistically activate transcription by binding to multiple adjacent sites and can counter-act Sp1 mediated synergistic transactivation (Yu *et al.* 2003). However, it has been shown that Sp3 can substitute Sp1 in the synergistic mechanism, via the BTD as domain described by Athanikar and co-workers (Athanikar *et al.* 1997).

Sp4 has only one isoform of approximately 110 kDa and can either transactivate or silence gene transcription (Hagen *et al.* 1995, Ramos *et al.* 2009). The D domain that is responsible for the ability of Sp1 to form multimeric complexes is lacking in both Sp3

and Sp4. These TFs are therefore thought to be unable to synergistically transactivate its targets. However, Sp4 has been shown to directly interact with Sp1 forming heterotypic interactions, which can lead to an enhancement of the Sp1 activity when mediated through at least two Sp-response elements (Hagen *et al.* 1995).

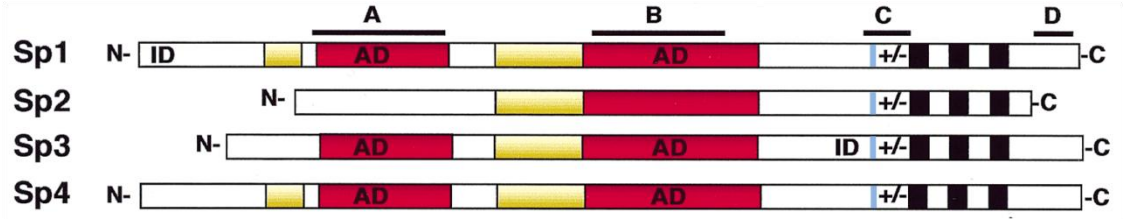

Figure 1.8. Schematic representation of the four human Sp-family members Sp1, Sp2, Sp3 and Sp4. Sp3 refers to the full length isoforms. Yellow boxes indicate regions of the proteins which are rich in serine/ threonine residues and red boxes regions of the proteins which are rich in glutamine. Blue boxes represent the BTD box. The region preceding the first zinc finger (+/−) represents a highly charged region. The black boxes represent the zinc fingers. Lines above Sp1 protein indicate the extent of four domains (A, B, C and D) which contribute to the transcriptional properties of the Sp proteins. Activation (AD) and inhibitory domains (ID) are indicated. *Adapted from Suske 1999.*

Sp knockout mice have revealed overlapping but distinct functions. Knocking out Sp1 and Sp3 was shown to be lethal. In addition, knocking down one of these factors will decrease the levels of both factors (Marin *et al.* 1997, Bouwman *et al.* 2000). Furthermore, heterozygous $Sp1^{+/-}$ $Sp3^{+/-}$ mice are also not viable, which suggests that the right amount of both Sp1 and Sp3 transcription factors is necessary in maintaining appropriate gene expression programs (Kruger *et al.* 2007). As expected, the defects in Sp4 knock-out mice are more pronounced in the nervous system, furthermore two-thirds of the Sp4 knockout mice die within a few days of birth and the surviving animals are smaller in size when compared to their littermates (Supp *et al.* 1996; Zhou *et al.* 2005).

Although nothing is known about PTMs that regulate Sp4 activity, Sp1 and Sp3 have been shown to be regulated by several PTMs. Sp1 can be phosphorylated by at least nine different kinases, it can be acetylated, glicosylated, ubiquitylated, SUMOylated and ADP-ribosylated (reviewed in Wierstra 2008). The major determinant of Sp3 activity was found to be SUMOylation (Sapetschnig *et al.* 2004), nevertheless this protein can also suffer phosphorylation, glicosylation and acetylation (Li & Davie 2010). All of these PTMs alter Sp proteins activity and determine their role as repressors or as activators of gene expression.

Despite the similarities between these TFs and the fact that they recognize the same response elements albeit with different affinities, it has been extensively shown that different genes are regulated by the different members of this family of transcription factors or by the different ratio between the Sp proteins present at the proximal promoters of target genes. This demonstrates that the Sp proteins, like other TFs, are part of intricate gene regulatory networks and that PTMs and protein-protein interactions play an important role in regulating gene expression by modulating the Sp TFs activity and directing their specific function.

OBJECTIVES

The body of work presented in this dissertation focuses on the characterization of the molecular mechanisms underlying the brain-specific expression of the human cytochrome P450, CYP46A1. This cholesterol 24-hydroxylase is responsible for the majority of brain cholesterol turnover, nevertheless, despite its physiological importance, the regulatory networks that govern its expression had not been characterized.

The cloning of 5kb of the *CYP46A1* 5' flanking region reveled an extremely GC-rich promoter lacking a consensus TATA box and rich in putative Sp binding sites. Therefore, we hypothesize that the Sp family of transcription factors might play an important role in the basal expression of this gene.

Afterwards, we aimed to identify a human cell model that expresses high levels of CYP46A1, in order to confirm our results at the chromatin level and subsequently to validate this cell system as a valuable tool for the study of cholesterol homeostasis in human neurons.

Since the bioinformatic analysis of the 5'flanking region demonstrated that the *CYP46A1* proximal promoter region is located in a predicted CpG island, we have raised the question if epigenetic mechanisms, such as DNA methylation, could be involve in the cell-specific expression of this gene.

The experimental approaches used involve a wide range of cell and molecular biology methods and were designed to answer the following questions:

1. Which are the regulatory elements and factors involved the *CYP46A1* brain specific expression?

2. Which is the particular role of the Sp family of transcription factors in the regulation of the *CYP46A1* gene?

3. Are differentiated Ntera2/clone D1 (NT2) human teratocarcinoma cells a suitable model to study the human *CYP46A1* regulation?

4. Are differentiated NT2 a valuable tool for the study of cholesterol homeostasis in human neurons?

5. Is the role of DNA methylation important in the tissue-specific expression of *CYP46A1*?

Taken together, the results presented herein are a significant contribution to the definition of the regulatory mechanisms that control human *CYP46A1* transcription and may prove useful in unraveling potential therapeutic agents that can modulate this key enzyme gene expression and thus control brain cholesterol metabolism in pathophysiological situations.

TRANSCRIPTIONAL REGULATION OF THE HUMAN *CYP46A1* BRAIN-SPECIFIC EXPRESSION BY SP TRANSCRIPTION FACTORS

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2.1 Abstract

Brain defective cholesterol homeostasis has been associated with neurologic diseases, such as Alzheimer's and Huntington's disease. The elimination of cholesterol from the brain involves its conversion into 24(*S*)-hydroxycholesterol by CYP46A1, and the efflux of this oxysterol across the blood-brain barrier. Herein, we identified the regulatory elements and factors involved the human *CYP46A1* expression. Functional 5'deletion analysis mapped a region spanning from nucleotides -236/-64 that is indispensable for basal expression of this TATA-less gene. Treatment of SH-SY5Y cells with mithramycin A resulted in a significant reduction of promoter activity, suggesting a role of Sp family of transcription factors in *CYP46A1* regulation. Combination of Sp1, Sp3, and Sp4 over-expression studies in *Drosophila* SL2 cells, and systematic promoter mutagenesis identified Sp3 and Sp4 binding to four GC-boxes as required and sufficient for high levels of promoter activity. Moreover, Sp3 and Sp4 were demonstrated to be the major components of the protein-DNA complexes observed in primary rat cortical extracts. Our results suggest that the cell-type specific expression of Sp transcription factors – substitution of Sp1 by Sp4 in neurons – is responsible for the basal expression of the *CYP46A1* gene. This study delineates for the first time the mechanisms underlying the human *CYP46A1* transcription and thereby elucidates potential pathways underlying cholesterol homeostasis in the brain.

2.2 Introduction

The brain, although composing < 5% of the total body mass contains approximately 25% of all the unesterified body cholesterol. Cholesterol metabolism in the central nervous system (CNS) is distinct from that in other tissues. Indeed, because of the efficiency of the blood–brain barrier (BBB), the brain is unable to take-up cholesterol from the circulation and relies almost completely on de novo synthesis. Cholesterol in the adult brain is largely metabolically inert; however, a small fraction of the pool turns over each day. The major mechanism by which cholesterol is metabolized in the brain is by conversion to 24(*S*)-hydroxycholesterol (24OHC), a more polar sterol that can traverse the BBB, enter circulation, and subsequently be cleared by the liver (Bjorkhem *et al.* 1998). The flux of this oxysterol is believed to be the main mechanism by which the brain facilitates the removal of cholesterol. The 24OHC is a ligand for the liver X receptor (LXR), a nuclear hormone receptor that regulates the expression of many genes involved in lipid metabolism. LXRα/β null mice show a variety of CNS defects upon aging including lipid accumulation, astrocyte proliferation, and disorganized myelin sheaths (Wang *et al.* 2002).

The enzyme responsible for the conversion of cholesterol to 24OHC was identified as a brain-specific cytochrome P450, the CYP46A1 (Lund *et al.* 1999). Knock-out mice lacking Cyp46a1 have an approximately 65% reduction in brain cholesterol excretion, but this decrease is compensated for by a reduction in the de novo synthesis, such as steady-state levels of cholesterol remain constant (Lund *et al.* 2003). On the other hand, Cyp46a1 null mice exhibit severe deficiencies in spatial, associative, and motor learning, and hippocampal long-term potentiation (Kotti *et al.* 2006).

Cholesterol 24-hydroxylase mRNA and protein are expressed at low levels in the liver and testis and at much higher levels in the adult brain. Immunohistochemical and in situ mRNA hybridization experiments indicate that Cyp46a1 is expressed in pyramidal neurons of the mouse cerebral cortex, Purkinje cells of the cerebellum, neurons of the thalamus, dentate gyrus, and hippocampus but not in support cells or in the white matter of the adult brain (Lund *et al.* 1999). CYP46A1 mRNA and protein are both expressed in the brain at birth, and 24OHC is detected in the serum on postnatal day 1. 24OHC levels in the serum peak between days 1 and 21 during the time of myelination, and thereafter, levels of this oxysterol gradually increase in the brain. In accordance, in humans, at the

age of about 1 year, expression levels of CYP46A1 reach a steady state, which is maintained during adulthood (Lund *et al.* 1999).

An increasing number of studies suggest that CYP46A1 affects the pathophysiology of Alzheimer's disease (AD). Indeed, while CYP46A1 expression is restricted to neurons in healthy brains, a relative high protein concentration has been reported in glial cells of AD brains (Bogdanovic *et al.* 2001). Moreover, several groups have described an association between genetic variants of CYP46A1 and AD (Johansson *et al.* 2004, Papassotiropoulos *et al.* 2003). Changes in the flux of 24OHC from the brain into the circulation have also been reported in patients with AD (Papassotiropoulos *et al.* 2002). The 24OHC is able to induce the expression of ATP binding cassette transporters (ABC) A1, G1 and apoE in astrocytes and to elevate apoE-mediated cholesterol efflux in astrocytoma cells (Abildayeva *et al.* 2006). Moreover, 24OHC inhibits amyloid β secretion in primary cultures of cortical neurons (Brown *et al.* 2004).

Although significant evidences have been presented that the deregulation of cholesterol balance may be related to the onset of neurological diseases, the molecular mechanisms that underlie the brain-specific expression of the human *CYP46A1* gene have never been described. In addition, little is known about the transcriptional modulation of this gene by endogenous or exogenous signals. The recently cloned *CYP46A1* 5'-flanking region was found to be rich in putative Sp binding sites (Ohyama *et al.* 2006).

Transcription factors belonging to the family typified by Sp1 are involved in the expression of a large number of genes, including most of those known as housekeeping genes. Spl, Sp3 and Sp4 are closely related members of the Sp gene family encoding proteins with very similar structural features. Most significantly, the DNA binding domains of Spl, Sp3 and Sp4 are highly conserved, formed of three conserved Cys2His2 zinc fingers (Suske 1999), that recognize the GC box (GGGGCGGGC) and the GT motif (GGGTGTGGC) with identical affinities (Hagen *et al.* 1992).

Within this family, Sp1 and Sp3 are ubiquitously expressed in mammalian cells while Sp4 expression is highly enriched in the brain (Hagen *et al.* 1992). Although, Sp1, Sp3 and Sp4 present structure similarities, comparison of the Sp knockout mice phenotypes have revealed overlapping as well as distinct functions (Bouwman *et al.* 2000). Indeed, null mutation of Sp1 in mouse is embryonic lethal (Marin *et al.* 1997). Similarly, Sp3 null mice die soon after birth (Bouwman *et al.* 2000). As expected, the abnormalities arising from Sp4 ablation are most apparent in the nervous system (Supp *et al.* 1996, Zhou *et al.* 2005).

In the present study, we aimed at identifying regulatory elements and factors involved in basal *CYP46A1* expression. Moreover, since the *CYP46A1* promoter was found to contain multiple potential Sp binding sites, the particular role of the Sp family of transcription factors in the *CYP46A1* brain-specific expression was evaluated.

2.3 Methods

2.3.1 Cloning of the 5'-upstream region of the human *CYP46A1* gene

The 5'-upstream region sequence of the human *CYP46A1* gene was identified with the Basic Local Alignment Search Tool (BLAST) using the human CYP46A1 mRNA sequence (GenebankTM accession number NM_006668). A 4.8-kb fragment of human *CYP46A1* promoter was cloned in pCR®-2.1TOPO® vector (Invitrogen Corporation, California, USA) after amplification by PCR using human genomic male DNA (Promega Corporation, Madison, WI, USA) as a template and the Expand Long Template PCR System (Roche Diagnostics GmbH, Penzberg, Germany). The primers used to amplify a 4765 bp region between nucleotides -4828 and -64 are listed in Table 2.1 (primers hCYP46PromF2 and hCYP46PromR2; +1 refers to the A of the initiation methionine). The insert of the recombinant plasmid harboring the *CYP46A1* promoter region encompassed by nucleotides -4828 and -64 was sequenced by primer walking and named promCYP46-pCR2.1.

2.3.2 Plasmids

Several different fragments derived from the 5' flanking region of the human *CYP46A1* gene were subcloned in the luciferase expression vector pGL2 Basic vector (Promega) using standard recombinant technology methods. The promCYP46-pCR2.1 recombinant was digested with *Kpn* I/ *Xho* I (1290bp), *Mlu* I/ *Xho* I (353bp), *Sac* I/ *Xho* I (209bp) and each of the restriction fragment were subcloned in the pGL2 reporter plasmid, generating plasmids 1.2pGL2 (-1219 to -64; +1 refers to the A of the initiation methionine), 0.3pGL2 (-417 to -64) and 0.2pGL2 (-271 to -64). Two fragments of the human *CYP46A1* proximal promoter were amplified with forward primers 120XmaIf and SP-RE-B and the reverse primer XhoIr (Table 2.1) containing a *Xho* I overhang site, using *Pfu* polymerase (Stratagene, CA) and the promCYP46-pCR2.1 plasmid as template. The PCR amplified fragments were subcloned in pGL2 reporter plasmid into the *Sma* I

and *Xho* I sites, generating recombinants 0.12pGL2 (-236 to -64) and 0.09pGL2 (-152 to -64).

The rat *CYP46A1* proximal promoter was identified as described above using the rat CYP46A1 mRNA sequence (GenebankTM accession number XM_343108) and a 168 nucleotide fragment was amplified by PCR using rat genomic DNA and primers RatCYP46promf and RatCYP46promr (Table 2.1). The amplified fragment was subcloned in the pGL2 reported construct into the *Sma* I and *Xho* I sites, generating construct rCYP46Prom (-226 to -54; +1 refers to the A of the initiation methionine).

2.3.3 Cell culture

SH-SY5Y (human neuroblastoma) and U118 (human glioma) cell lines were maintained in low glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich Inc., St Louis, MO, USA), while HeLa (cervix adenocarcinoma) was cultured in high glucose DMEM (Sigma) at 37 ºC in humidified 5% CO_2. All media were supplemented with 10% heat inactivated fetal bovine serum (Biochrom KG, Berlin, Germany), 2mM L-glutamine (Sigma), 100 units/ml penicilline and 100µg/ml streptomycin (Sigma), with the exception of U118 culture medium that was supplemented with 4 mM L-glutamine.

Cultures of *Drosophila melanogaster* Schneider cell line (SL2) were maintained in Schneider medium (Sigma) supplemented with 10% heat inactivated fetal bovine serum, 50 units/ml penicillin and 50 µg/ml streptomycin at 25 ºC and atmospheric CO_2.

Neurons were isolated from fetuses of 17–18 day pregnant Wistar rats, as previously described (Silva *et al.* 2002). Astrocytes were isolated from 2-day-old rats as previously described (Blondeau *et al.* 1993), with minor modifications (Silva *et al.* 1999). Animal care followed the European Legislation on Protection of Animals Used for Experimental and Scientific Purposes (EU directive L0065, 22/07/2003).

2.3.4 Transactivation assays

To minimize variations in transfection efficiency, replicates were transfected, in single batch suspension with FuGENE 6 (Roche) according to the manufacturer's instructions. Plates containing 150 000 cells were co-transfected with 0.5 µg of the reporter plasmid and different amounts of each expression vector. Cells were inoculated in 24-well plates

and maintained for 48h. These cells were harvested and lysed in reporter lysis buffer (Promega). Cell extracts were assayed for luciferase and β-galactosidase activity (β -Gal Reporter Gene Assay, Roche). The SL2 cell extracts were normalized for the total amount of protein (BCA reagent, Pierce, Rockfort, IL) prior to performing the luciferase assay, since Sp expression vectors were shown to severely induce β -galactosidase expression in transfected cells.

2.3.5 Site-directed mutagenesis

The plasmids 0.09pGL2 and 0.12pGL2 served as the template for site-directed mutagenesis using the QuickChange Site Directed Mutagenesis Kit (Stratagene). All reactions were performed according to the protocol provided by the manufacturer. The MD90CYP46 oligonucleotide (Table 2.1 - mutated bases underlined) was used for site-directed mutagenesis of the SP-RE-A and SP.RE-B binding sites, while oligonucleotide MDSPRE-C was used for mutagenesis of SP-RE-C and MD120SPRE-D for SP-RE-D site.

2.3.6 *CYP46A1* expression analysis

Total cell RNA was extracted using Trizol Reagent (Invitrogen) following manufacturer's instructions. RNA was finally resuspended in nuclease-free water (Promega), and quality of the RNA was checked on a 2.2 M formaldehyde/MOPS 1.2% agarose gel. Total RNA from human brain was purchase at BD Biosciences, Palo Alto, US. RT-PCR (Access RT-PCR System, Promega) was performed with 1 µg of total RNA with the following primers (Table 2.1): CYP46A1 (RTCYP46f and RTCYP46r); human β-actin (hACTINf and hACTINr), rat β-actin (rACTINf and rACTINr). Reaction conditions were as follows: 45 °C for 45 min, 94 °C for 2 min, followed by 35 cycles of denaturation at (94 °C, 30 s), annealing (55 °C, 1 min), and extension (68 °C, 1 min), with a final extension (68 °C, 7 min).

2.3.7 Nuclear extracts

Nuclear extracts were prepared as described by Schreiber and co-workers (Schreiber *et al.* 1989). Recombinant Sp proteins used in the gel mobility-shift assays were obtained by

transfecting 8 x 10^6 SL2 cells with 10 µg of pPac-Sp1, pPac-Sp3FL and pPac-Sp4. Cells were seeded in 100mm plates and maintained for 48 h for nuclear extract preparation.

2.3.8 Western blot analysis

Twelve micrograms of nuclear protein were subject to 8% SDS-PAGE gels and electroblotted onto Immobilon P (Millipore, Bedfore, MA). After visualisation of the transferred proteins by amido black staining, the membranes were incubated with anti-Sp1 (PEP-2), anti-Sp3 (D-20), or anti-Sp4 antibody (V-20) from Santa Cruz Biotechnology. Results were quantified using the Quantity One version densitometry analysis software (Bio-Rad Laboratories).

2.3.9 Electroforetic mobility shift assay (EMSA)

Double-stranded DNA probes and competitors were generated by annealing of complementary single-stranded oligonucleotides Sp-cons, SP-RE-A, SP-RE-B, SP-RE-C, mSP-RE-C, SP-RE-D, mSP-RE-D, SP-RE-E, SP-RE-F, SP-RE-G, SP-RE-H, SP-RE-I, and SP-RE-J (see Table 2.1). DNA (6 pmol) was end-labeled with [γ^{32}P] dATP using T4 polynucleotide kinase, and unincorporated nucleotides were removed by Sephadex G25 filtration. The binding reactions were performed at 4 ºC in a total volume of 20 µl and contained: 2-5µg of nuclear extract, 10 mM of HEPES buffer, pH 8.0; 0.1 mM EDTA; 2 mM DTT; 17.5% glycerol; 40 mM spermidine; 40 mM MgCl2; 0.5 µg of dIdC; 1 µg salmon sperm DNA; and 0.5-2 ng of oligonucleotide probe. In competition assays excess unlabeled oligonucleotide was pre-incubated (30 min), prior to incubation with each probe for additional 20 min. Super-shift reactions, were performed with 1 µl of anti-Sp1 (PEP-2, Santa Cruz Biotechnology), of anti-Sp3 (D-20, Santa Cruz Biotechnology), or of anti-Sp4 antibody (V-20, Santa Cruz Biotechnology), which was added to the reaction media, that were kept on ice for 30 min before addition of the probe. Protein-DNA complexes were resolved on 5% non-denaturing PAGE gels (29:1 acrylamide: bisacrylamide) in 0.5 X Tris-borate buffer (45 mM Tris-borate, 1mM EDTA). The gels were eletrophoresed for 2 h 30 m, at 30 mA.

Table 2.1. Sequence of oligonucleotides used in this study: Putative Sp responsive elements in bold, mutated bases in small caps, and the position of the restriction site used in subsequent cloning are underlined.

Oligonucleotide	Cloning	Mutagenesis	EMSA	RT-PCR	Sequence (5'→3')
hCYP46PromF2	X				GAGGTTCAAGGAAGCTGGAAAC TGGGTTCCAGTCCTG
hCYP46PromR2	X				GAGCCGACTCAGCTGTCAG
120XmaIf	X				AAACCCGGGGGAGGGCCCGG
XhoIr	X				TGACAGCTGAGTCGGCTCGAGC CG
RatCYP46promf	X				CGGGGGCGGGTCCTGAGAGC
RatCYP46promr	X				CGGCTCGACCGACTAGCTGTCA GC
MD90CYP46		X			CATAACCCCGGCG**GGttCttAGC**C GAG**CGttCttGGA**GGGTGCTGGG TC
MD120SPRE-C		X			GGACTGTGCGCCC**TGttAtGtGT**C GGAGTCGCG
MD120SPRE-D		X			CCCAAACCCG**GGttAtGtCC**CGG AGCGCCGGAGC
Sp-cons			X		ATTCGAT**CGGGGCGGGG**CGAG C
SPRE-A			X		CCGAG**CGGGCGGGGA**GGGTG
SPRE-B	X		X		CGGCG**GGGGCGGAGC**CGAGC
SPRE-C			X		CGCCC**TGGGAGGGGT**CGGAG
mSPRE-C			X		CGCCC**TGttAtGtGT**CGGAG
SPRE-D			X		AAACG**GGGGAGGGCC**CGGAG
mSPRE-D			X		AAACG**GGttAtGtCC**CGGAG
SPRE-F			X		AGGTT**GGGGCGGAGC**TCTGG
SPRE-G			X		GGGCT**GGGGCGGTGT**GGAGG
SPRE-H			X		TGCTC**GGGGTGGGGC**CTGGG
SPRE-I			X		AGGTG**GGGGCGGAGC**CTGGG
SPRE-J			X		CTGCT**GGGGCGGACC**TGAGT
RTCYP46f				X	TCA GTC ATC GTC ACG AGT CC
RTCYP46r				X	CTG GTC TCC ATC CCA AAA GC
hACTINf				X	GCA CCA CAC CTT CTA CAA TGA GC
hACTINr				X	AAT GTC ACG CAC GAT TTC CCG C
rACTINf				X	TATGGAGAAGATTTGGCACC
rACTINr				X	CCACCAATCCACACAGAGTA

2.3.10 Statistical analysis

Statistical analysis was performed using the Student's *t*-test and the ANOVA one-way test with the Tukey HSD post-hoc test or the Tukey HSD for unequal N (Spjotvoll/Stoline test). All analysis was performed using the STATISTICA (data analysis software system), version 7.1 StatSoft, Inc. (2006).

2.4 Results

2.4.1 Cloning of the 5´-upstream region of the human *CYP46A1* gene

A 4765bp fragment of the human *CYP46A1* gene (between nucleotides -4828 and -64; +1 refers to the A of the initiation methionine), was identified with BLAST and amplified as described in section 2.3.1.

As previously described, like in many neuronal genes, the *CYP46A1* 5'-flanking region was found to be GC-rich and lacking a typical CAAT or TATA boxes (Ohyama *et al.* 2006). The most striking feature of the *CYP46A1* proximal promoter was the presence of 10 putative Sp-binding sites in the region encompassed by nucleotides -417 to -64 (Figure 2.1 A).

The *CYP46A1* reporter plasmids and the parental pGL2basic vector were transfected into SH-SY5Y (human neuroblastoma) and into U118 (human glioma) cells, which express low levels of endogenous CYP46A1 (Ohyama *et al.* 2006), and into a cell line in which CYP46A1 mRNA is undetectable - HeLa (cervix adenocarcinoma) (Figure 2.1 B). Surprisingly, *CYP46A1* reporter constructs presented high luciferase activities even in the cell lines where CYP46A1 mRNA could not be detected (Figure 2.1 B). Significant differences were found between the luciferase activities of the different constructs, namely in SH-SY5Y cells (ANOVA one-way test: $F = 242.3$, $df = 5$, $p < 0.001$). Post-hoc comparisons revealed that significant differences were found between reporter constructs pGL2 and 0.09pGL2 (Tukey HSD $p < 0.001$), and between 0.09pGL2 and 0.12pGL2 (Tukey HSD $p < 0.05$), suggesting that basal proximal promoter is located between nucleotides -236 and -64.

Since the *CYP46A1* proximal promoter has several GC boxes which could represent binding sites for the Sp family of transcription factors, we have investigated whether Sp family members are important in the regulation of this gene.

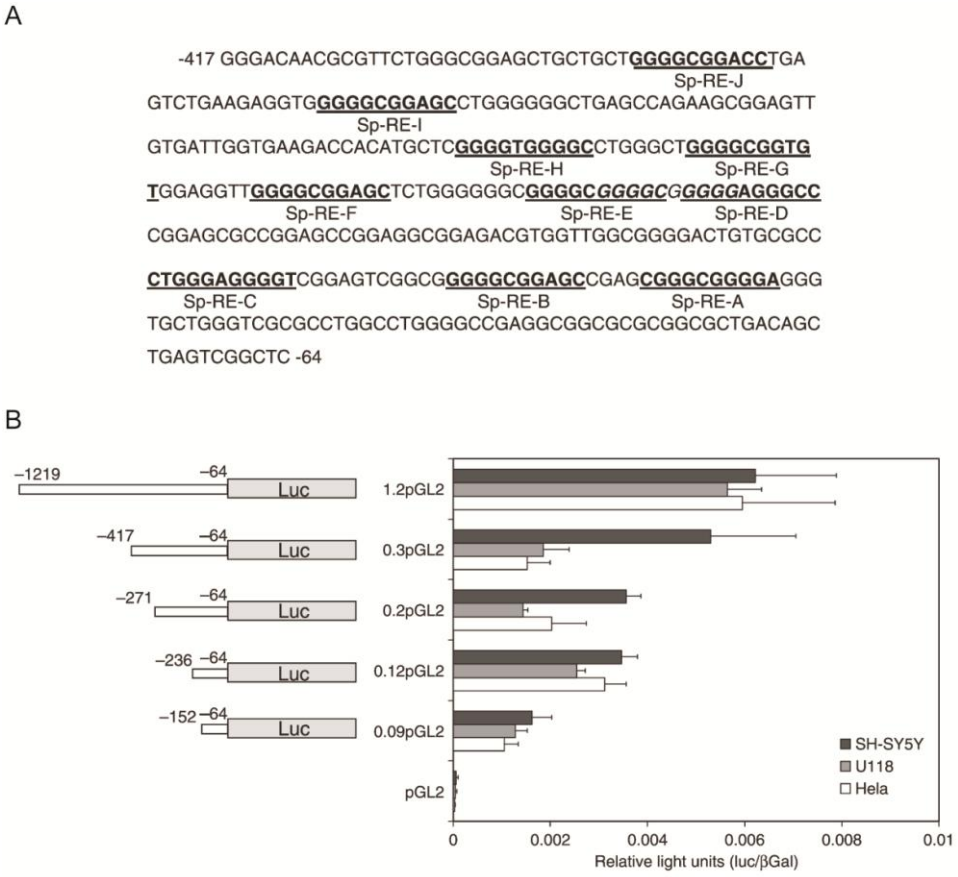

Figure 2.1. Functional deletion analysis of the human *CYP46A1* gene promoter. A) Nucleotide sequence of the human *CYP46A1* 5' flanking region (- 417 to -64; +1 refers to the A of the initiation methionine). Putative Sp binding sites are underlined. B) Basal transcriptional activity of the human *CYP46A1* promoter in different cell lines. Progressive 5' deletion constructs were transiently transfected into HeLa, U118 and SH-SY5Y cell lines. Transfections were carried out using 0.5 µg of the *CYP46A1* reporter construct or the empty pGL2 vector. Normalized luciferase activities were expressed as mean values ± SD of duplicates for a minimum of three experiments.

2.4.2 Basal activity of the *CYP46A1* promoter

SH-SY5Y cells were transfected with the 1.2pGL2 construct and treated with different dosages of Sp binding inhibitor mithramycin A. Mithramycin A has been shown to inhibit the ability of Sp to bind to DNA and thus act as a transcriptional inhibitor of gene expression (Chatterjee *et al.* 2001, Ray *et al.* 1989). Treatment with mithramycin A resulted in a significant reduction of *CYP46A1* promoter activity in a dose-dependent manner (ANOVA one-way test: $F = 14.47$, $df = 3$, $p < 0.001$) (Figure 2.2). Addition of 25, 75 and 125 nM mithramycin A for 24 h decreased the promoter activity to 71.8%, 46.9% and 21.7% respectively, as compared to untreated 1.2pGL2. The post-hoc comparisons revealed that significant differences were found between the untreated cells

and cells treated with 75 and 125 nM of mithramycin A (Tukey HSD: 75 nM, $p < 0.05$; 125 nM, $p < 0.01$).

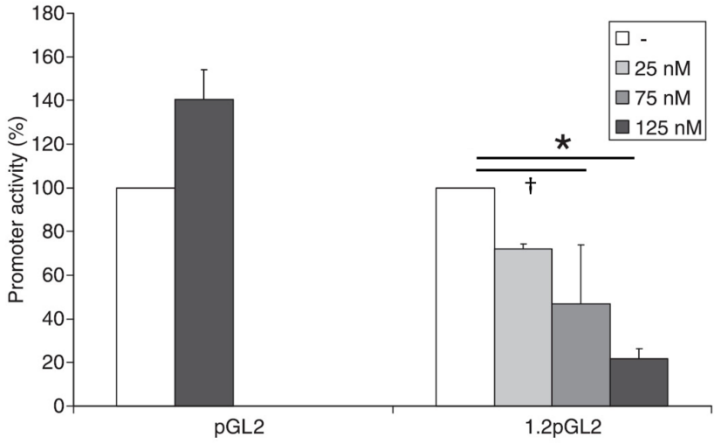

Figure 2.2. CYP46A1 promoter activity is repressed by mithramycin A. Transfections were carried out in SH-SY5Y cells using 0.5 µg of the 1.2pGL2 reporter construct or the empty pGL2 vector. Two hours after transfection, cells were treated with vehicle or with 25, 75, or 125 nM of mithramycin A. Cells were harvested 24 h after the treatment and the resultant luciferase activity was measured. The normalized luciferase activity of cells transfected with pGL2 or 1.2pGL2 and vehicle-treated was set to 100%, and the activities of treated samples calculated and plotted as a percentage of this value. Results were expressed as mean ± SD of duplicates for a minimum of three experiments (†$p < 0.05$; *$p < 0.001$, significant differences due to mithramycin A treatment).

2.4.3 Identification of Sp-responsive sequences in the *CYP46A1* gene promoter

To further examine the role of Sp proteins in *CYP46A1* gene transcription we have investigated the individual role of Sp1, Sp3 and Sp4 transcription factors, employing *Drosophila* SL2 cells. These cells lack endogenous Sp factors and therefore allow investigation of gene regulation without interference of endogenous Sp proteins (Dennig *et al.* 1995).

Over-expression of the ubiquitously expressed Sp1 or Sp3 significantly increased the luciferase reporter gene activity of all the CYP46A1pGL2 recombinants (Figure 2.3 A1). The strongest transactivation levels, 18 and 24 fold were attained in the presence of the 0.12pGL2 and 0.2pGL2 constructs respectively (Student's *t*-test, $p < 0.001$). Deletion of the DNA segments -236 to -152 and -152 to -64 significantly reduced transactivation by Sp1 (ANOVA one-way test: $F = 72.48$, $df = 4$, $p < 0.001$) and Sp3 (ANOVA one-way test: $F = 72.48$, $df = 4$, $p < 0.001$). The post-hoc comparisons revealed that significant differences were found between pGL2 and each of the other reporter constructs (Tukey

HSD for unequal N: 0.09pGL2 $p < 0.05$, 0.12pGL2 $p < 0.001$; 0.2pGL2, $p < 0.001$; 0.3pGL2, $p < 0.001$), and between 0.09pGL2 and each of the other reporter constructs (Tukey HSD for unequal N: 0.12pGL2 $p < 0.001$; 0.2pGL2, $p < 0.001$; 0.3pGL2, $p < 0.001$). Moreover, transactivation of the 0.3pGL2 was shown to be dose-dependent with the Sp1 and Sp3 expression vector (Figure 2.3 A2).

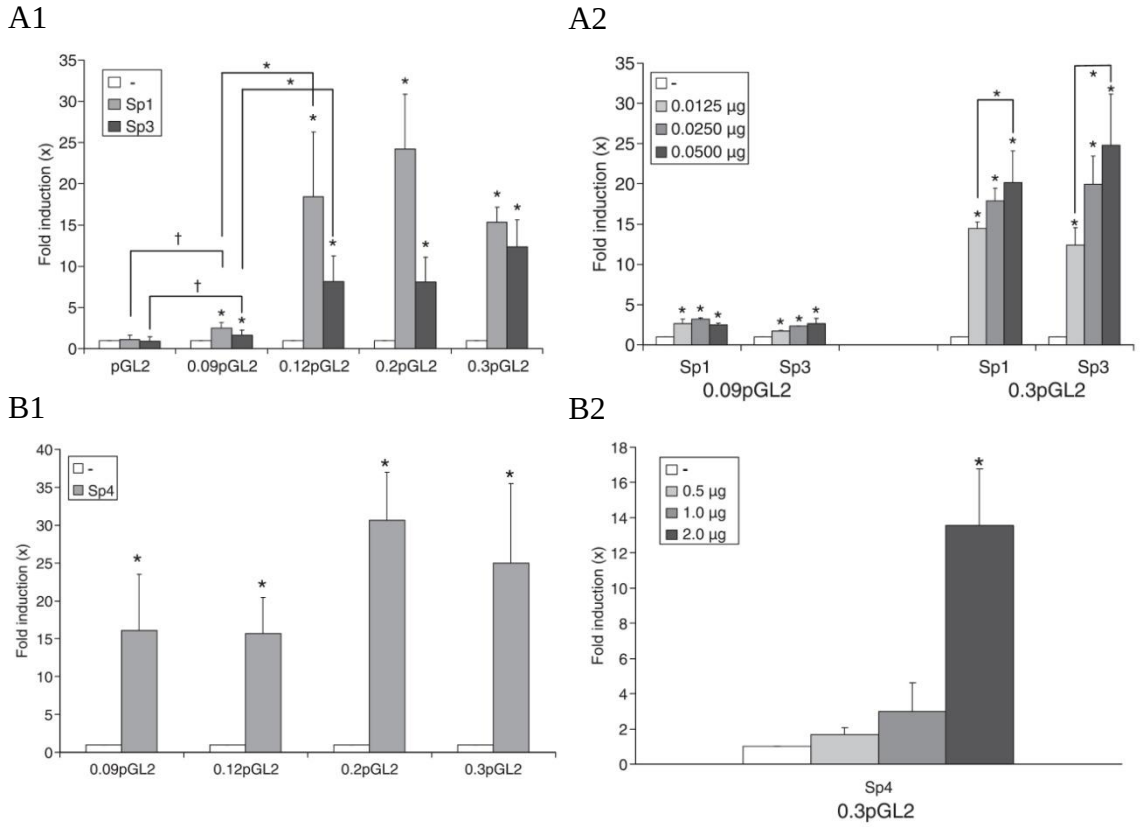

Figure 2.3. Effect of over-expressing Sp proteins on the human *CYP46A1* promoter in Sp-deficient *Drosophila* SL2 cells. Transactivation of the CYP46A1pGL2 reporter constructs by the ubiquitous Sp1 and Sp3 proteins. Co-transfection of 0.5 µg of the CYP46A1pGL2 reporter constructs with empty vector or with 0.025 µg (A1) or with increasing concentrations of pPacSp1 or pPacSp3 (0.0125–0.05 µg) (A2). Transactivation of the CYP46A1pGL2 reporter constructs by the brain-enriched Sp4 protein. Transfection of 0.5 µg of the CYP46A1pGL2 reporter constructs with empty vector or with 2 µg of pPacSp4 (B1), or with increasing concentrations of pPacSp4 (0.5–2 µg) (B2). Normalized luciferase activities were expressed as mean values ± SD of fold induction of duplicates for a minimum of three experiments (†$p < 0.05$; *$p < 0.001$).

The activation potential of the brain-enriched Sp4 protein was likewise assessed in SL2 cells and the results showed that the *CYP46A1* promoter was also highly transactivated by this transcription factor (15-fold activation) (Figure 2.3 B). Nevertheless, in contrast with what was found for Sp1 and Sp3, no significant differences

in the Sp4 activation levels were obtained between the different promoter deletion constructs. These results are in agreement with those of Hagen and co-workers (Hagen *et al.* 1992), which found that Sp4 is not able to act synergistically through adjacent binding sites. Moreover, transactivation of the 0.3pGL2 was also shown to be dose-dependent with the Sp4 expression vector (Figure 2.3 B2).

2.4.4 Functional cooperation among members of the Sp family in the activation of the *CYP46A1*

To investigate the combinatorial mechanisms of Sp proteins transcriptional regulation of the *CYP46A1* gene we transiently co-transfected SL2 cells with the 0.12pGL2 construct plus different combinations of Sp1, Sp3 and Sp4 expression vectors (Figure 2.4). The results showed that significant synergistic enhancement of transcriptional activation could be obtained upon co-expressing either Sp4-Sp1 (32-fold) or Sp4-Sp3 (38-fold). This important functional difference revealed the brain-enriched Sp4 protein as an important player in the regulation of the *CYP46A1* gene by Sp proteins.

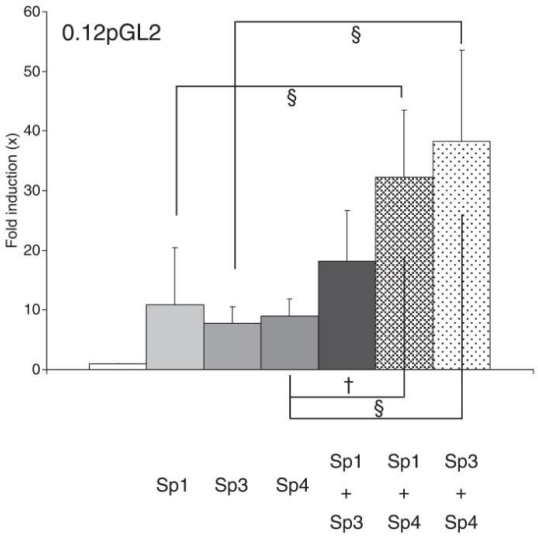

Figure 2.4. Sp4 functionally synergizes with Sp1 and Sp3 in activating the *CYP46A1* promoter. Co-transfection of 0.5 µg of the 0.12pGL2 reporter construct with empty vector or different combinations of pPacSp1, pPacSp3, and pPacSp4 expression vectors in SL2 cells. Normalized luciferase activities were expressed as mean values ± SD of fold induction of duplicates for a minimum of three experiments (†$p < 0.05$; §$p < 0.01$).

2.4.5 Characterization of the Sp binding sequences in the *CYP46A1* promoter region

We used gel mobility-shift assays with the goal of identifying active Sp binding sites within the *CYP46A1* locus. First, oligonucleotides encompassing the putative Sp responsive elements in -417 to -64 region of the *CYP46A1* promoter were synthesized. These were named Sp-RE-A, Sp-RE-B, and so forth (see Table 2.1 and Figure 2.1 A). HeLa cells nuclear extracts were used to characterize the binding activities of each Sp-RE site by electrophoretic mobility shift assay.

The specificity of the complex(es) formed when a Sp consensus oligonucleotide (Sp-cons) was used as a probe were verified by means of a supershift assay using an anti-Sp1 antibody (Figure 2.5 A: arrowhead). All Sp-RE oligonucleotides were able to compete for Sp1 binding to the Sp-cons, although with different affinities. Indeed, oligonucleotides Sp-RE-A, Sp-RE-C, Sp-RE-G, and Sp-RE-J were not able to completely prevent the formation of the Sp1/ Sp-cons complex at a 100-fold molar excess. The Sp1/ Sp-cons complex was not competed by an unrelated DNA sequence (Figure 2.5 A).

In order to prove that the Sp transcription factors were able to bind to the different Sp-REs, Sp1, Sp3, and Sp4 transcription factors were over-produced in SL2 cells, and the nuclear extracts used to characterize their binding activities to Sp-RE-A, Sp-RE-B, Sp-RE-C, and Sp-RE-D (Figure 2.5 B). Results have confirmed that the four binding sites were able to bind to each of the different Sp proteins, even though with apparently different affinities.

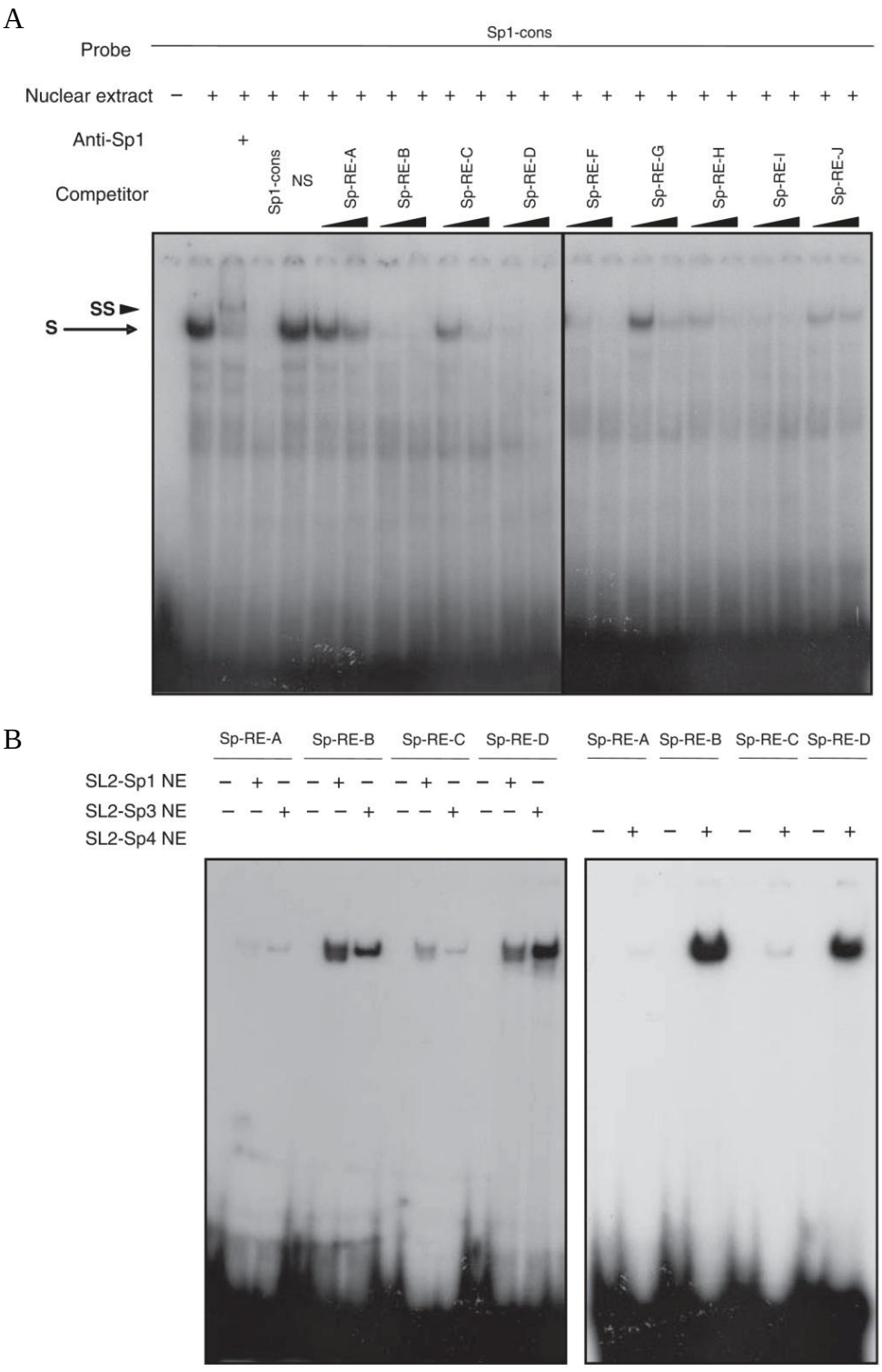

Figure 2.5. Characterization of the binding activities to Sp-RE sites. (A) EMSA was performed using HeLa cell nuclear extracts and a radiolabeled double-stranded oligonucleotide corresponding to the Sp consensus (Sp1-cons) as a probe. Competition experiments were performed by adding a 10- or 100-fold excess of unlabeled doublestranded oligonucleotides corresponding to cold-self, a non-specific tubulin sequence, and Sp-RE-A, -B, -C, -D, -F, -G, -H, -I and -J. Supershift experiments were performed using an anti-Sp1 antibody. (B) EMSA was performed with nuclear extracts of SL2 cells over-expressing Sp1, Sp3, and Sp4 proteins, using as probes radiolabeled double-stranded oligonucleotides corresponding to sites Sp-RE-A, -B, -C and -D.

2.4.6 Functional analysis of Sp binding sites in *CYP46A1* promoter by site directed mutagenesis

The functional relevance of sites Sp-RE-A, Sp-RE-B, Sp-RE-C, and Sp-RE-D for *CYP46A1* promoter activation was investigated by individual or combined mutagenesis of each site in the 0.09pGL2 and 0.12pGL2 constructs, followed by transfection into SH-SY5Y cells (Figure 2.6). As shown in Figure 2.6 A, individual or combined mutation of the four Sp-RE sites significantly reduced the activity of the 0.09pGL2 and 0.12pGL2 constructs (ANOVA one-way test: $F = 32.19$, $df = 8$, $p < 0.001$). The post hoc comparisons revealed no significant differences between the individual mutation of Sp-RE-C or Sp-RE-D sites, although protein–DNA binding reactions suggested different affinities of each of these sites for binding to Sp1/Sp3 (Figure 2.6B).

Combined mutation of Sp-RE-A and Sp-RE-B, or Sp-RE-C and Sp-RE-D, reduced the promoter activity to approximately 30–40% of the wt, while mutation of the four Sp-RE sites, reduced activity to approximately 15% of the wt.

The effectiveness of the introduced mutations in impairing Sp binding was tested by gel-shift competition assays with HeLa nuclear extracts and mutated oligomers. As demonstrated in Figure 2.6 B, the mutated oligonucleotides did not effectively compete with a Sp consensus probe for the binding to Sp1/Sp3 nuclear proteins.

Finally, we have co-transfected SL2 cells with the 0.12pGL2 mutants and pPac-Sp1, pPac-Sp3, and pPac-Sp4 expression vectors (Figure 2.6 C). As expected, results of individual or combined mutation of the four Sp-RE sites significantly reduced transactivation by Sp3 and Sp1 (ANOVA one-way test: $F = 32.19$, $df = 8$, $p < 0.001$). Nevertheless, the introduced mutations did not significantly reduce transactivation by Sp4 (data not shown), suggesting that other transcription factors might serve as a docking factor for Sp4. Taken together, these results strongly indicate that all four Sp-RE sites are functional elements of the *CYP46A1* promoter.

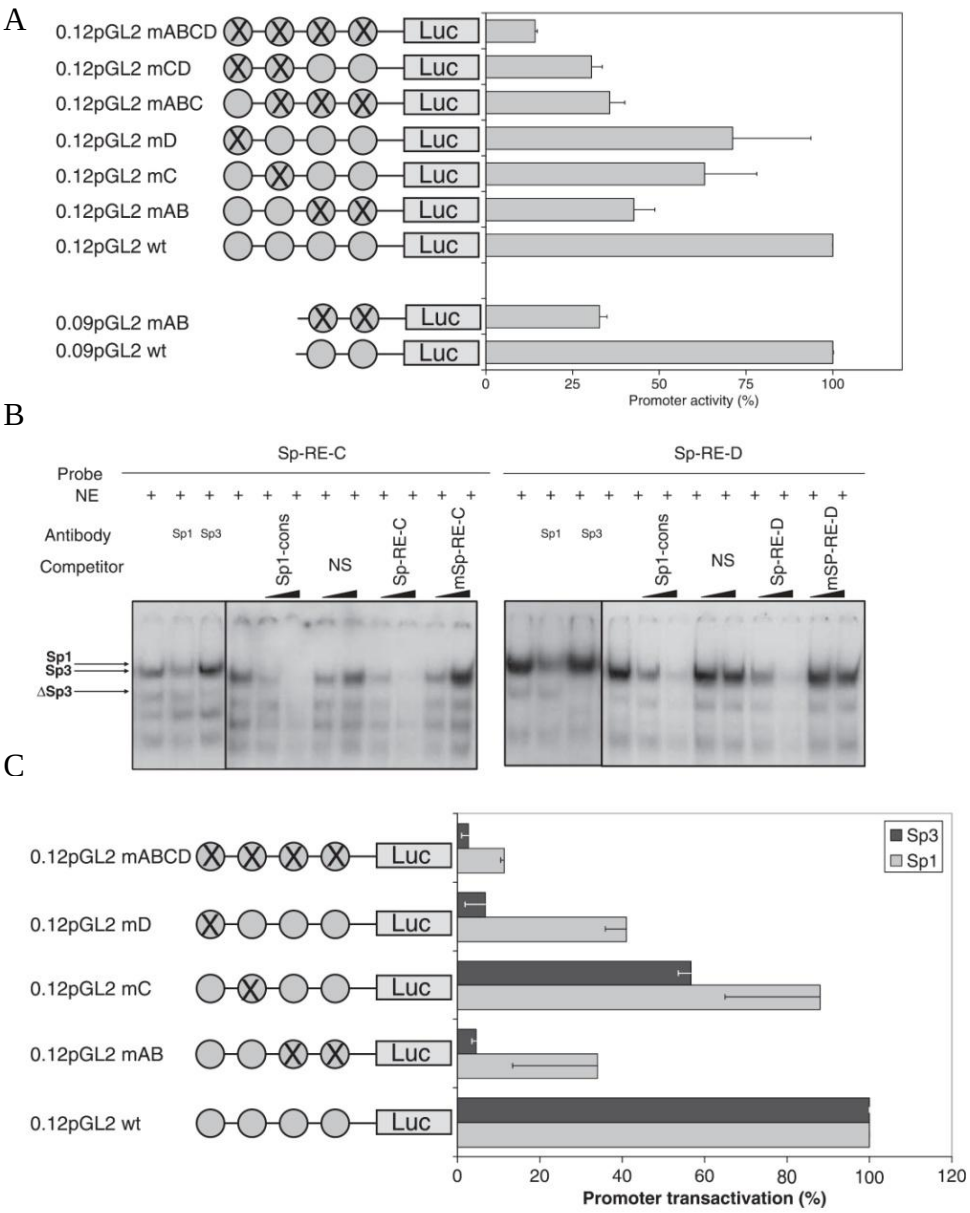

Figure 2.6. Mutational analysis of the Sp binding sites in the *CYP46A1* promoter using the 0.12pGL2 reporter plasmid altered by site-directed mutagenesis. (A) SH-SY5Y cells were transfected with 0.5 µg of wild-type (0.12pGL2 wt) or the different mutated promoter reporter plasmids (mAB, mC, mD, mABC, mCD mABCD). The normalized luciferase activity of the 0.12pGL2 wt was set to 100%, and the activities of the mutant plasmids were calculated and plotted as a percentage of this value. Results were expressed as mean values ± SD of duplicates for a minimum of three experiments. (B) EMSA was performed using HeLa cell nuclear extracts and a radiolabeled double-stranded oligonucleotide corresponding to the Sp-RE-C and Sp-RE-D as a probe. Competition experiments were performed by adding a 10- or 100-fold excess of unlabeled doublestranded oligonucleotides corresponding to Sp consensus (Sp cons), a non-specific tubulin sequence, cold-self and mutated Sp-RE-C or -D. Super-shift experiments were performed using an anti-Sp1 and anti-Sp3 antibodies. (C) SL2 cells were co-transfected with 0.5 µg of wild-type (0.12pGL2 wt) or the different mutated promoter reporter plasmids (mAB, mC, mD, mABC, mCD, mABCD) and with 0.025 µg of pPac-Sp1 or pPac-Sp3 expression vector). The normalized luciferase activity of the 0.12pGL2 wt construct transactivated by Sp1 or Sp3 was set to 100%, and the activities of the mutant plasmids were calculated and plotted as a percentage of this value.

2.4.7 Conservation among species of the Sp responsive elements in the *CYP46A1* promoter

We have cloned a 168 bp fragment of the rat *CYP46A1* proximal promoter (-226 to -54; +1 refers to the A of the initiation methionine), into the pGL2-basic vector (rat-CYP46A1pGL2), in order to assess if the Sp-responsive region was functionally conserved among species (Figure 2.7).

To investigate the individual role of Sp1, Sp3 and Sp4 transcription factors in the activation of the rat *CYP46A1* promoter we have over-expressed these proteins in SL2 cells transfected with the ratCYP46A1pGL2 reporter construct. A significant increase in the luciferase reporter activity was observed for samples transfected with pPac-Sp1, pPac-Sp3 and pPac-Sp4, demonstrating the functionality of the orthologous Sp-RE sites in rat *CYP46A1* promoter.

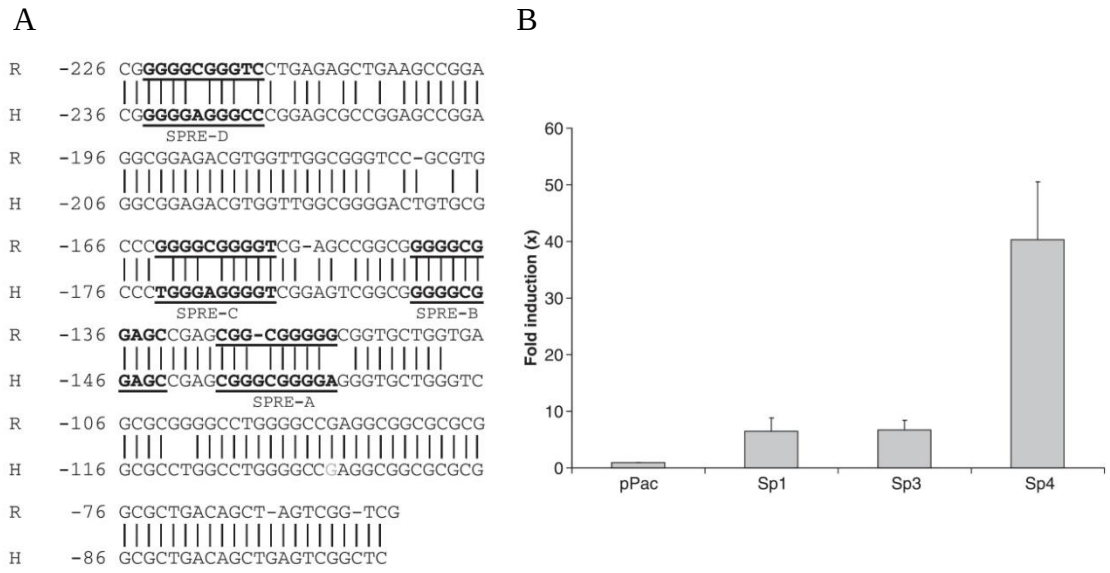

Figure 2.7. Transactivation of the rat *CYP46A1* promoter by Sp proteins in Sp-deficient *Drosophila* SL2 cells. (A) Comparison of the rat (R) and human (H) proximal promoter of the CYP46A1 genes. Putative Sp binding sites are underlined. Vertical dashed lines indicate sequence similarities. Numbers represent nucleotide positions relative to the first codon. (B) The ratCYP46A1pGL2 reporter (0.5 µg) was co-transfected with 0.025 µg of pPacSp1, 0.025 µg of pPacSp3, 1 µg of pPacSp4 or empty vector. Normalized luciferase activities were expressed as mean values ± SD of fold induction of duplicates for a minimum of three experiments.

2.4.8 Sp binding activity in primary cultures of cortical neurons and astrocytes

To determine which Sp proteins bind to the Sp-REs sites of the *CYP46A1* promoter in primary cultures of rat cortical neurons and astrocytes, specific antibodies against Sp proteins were added to the DNA-binding reactions using nuclear extracts from primary cell cultures (Figure 2.8).

In rat cortical neurons supershift analysis revealed that Sp3 and Sp4 were the major components of the complexes formed with the Sp-RE-D probe. Similar results were obtained with Sp-RE-B and Sp-RE-C (data not shown). Indeed, anti-Sp3 antibody, retarded complexes II and III, while the anti-Sp4 antibody retarded complex I. The anti-Sp1 antibody was unable to retard or deplete any of the complexes. In astrocytes nuclear extracts, the Sp proteins that bound the Sp-RE-D probe were Sp1 (complex I) and Sp3 (complex III). In this case, addition of the anti-Sp4 was unable to retard or deplete any of the complexes.

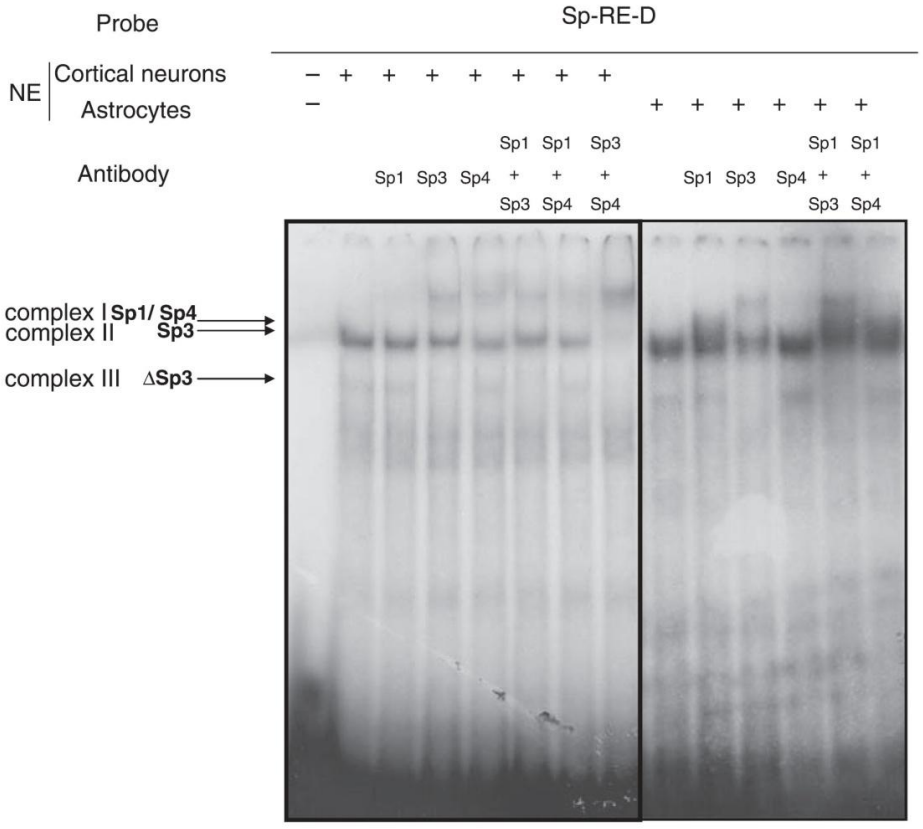

Figure 2.8. Sp proteins binding activity to the Sp-RE sites of the *CYP46A1* proximal promoter in nuclear extracts from primary cortical neurons and astrocytes. EMSA was performed using 5 µg of primary cortical neurons and astrocytes nuclear extract proteins and a radiolabeled double-stranded oligonucleotide corresponding to site Sp-RE-D as a probe. Super-shift experiments were performed with an anti-Sp1, -Sp3 and -Sp4-specific antibodies.

2.4.9 Correlation between Sp protein levels and CYP46A1 mRNA

The transactivation results obtained in SL2 cells transfected with different combinations of Sp1, Sp3, and Sp4 expression vectors have shown a significant synergistic enhancement of transcriptional activation by Sp4 when co-expressed with either Sp1 or Sp3. Moreover, supershift analysis revealed that Sp3 and Sp4 were the major components of the complexes formed with the Sp-RE probes in cortical neurons. These results suggested that *CYP46A1* expression levels may depend on the cell-specific differences in the expression levels of Sp proteins. Therefore, we have also determined the accumulation of the CYP46A1 mRNA in HeLa, U118, SH-SY5Y, and PC12 cells, as well as in primary cultures of rat cortical neurons and astrocytes. Total RNA from human brain and rat cortex were used as positive controls (Figure 2.9 A). In parallel, we have assessed the relative concentrations of Sp1, Sp3, and Sp4 protein levels (Figure 2.9 B).

RT-PCR analyses have shown that the human-derived cell lines present undetectable (HeLa) or extremely low levels of CYP46A1 mRNA (U118, SH-SY5Y). These results were confirmed by real-time quantitative RT-PCR (data not shown). As expected, the highest CYP46A1 mRNA levels were detected in primary cultures of rat cortical neurons (Figure 2.9 A). Although *in vivo* Cyp46a1 was reported to be expressed only in neurons (Lund *et al.* 1999), we could detect high levels of CYP46A1 mRNA in primary cultures of rat astrocytes.

As expected, western blot analysis exhibited distinctive levels of Sp1, Sp3, and Sp4 proteins in the different cells tested (Figure 2.9 B). Interestingly, an inverse correlation could be found between the Sp1/total Sp ratio and the CYP46A1 mRNA levels. These results were in agreement with our *in vitro* results which showed that the highest transactivation of the *CYP46A1* proximal promoter was achieved upon co-transfection of Sp3 and Sp4 expression vectors. On the other hand, the only cell line where CYP46A1 mRNA was undetectable (HeLa) presented the lowest levels of Sp4. Nevertheless, there was no correlation between the levels of Sp4 and CYP46A1 mRNA in continuous cell lines, which suggests that other transcription factors or epigenetic modifications of this GC-rich promoter might also be important for *CYP46A1* basal expression levels.

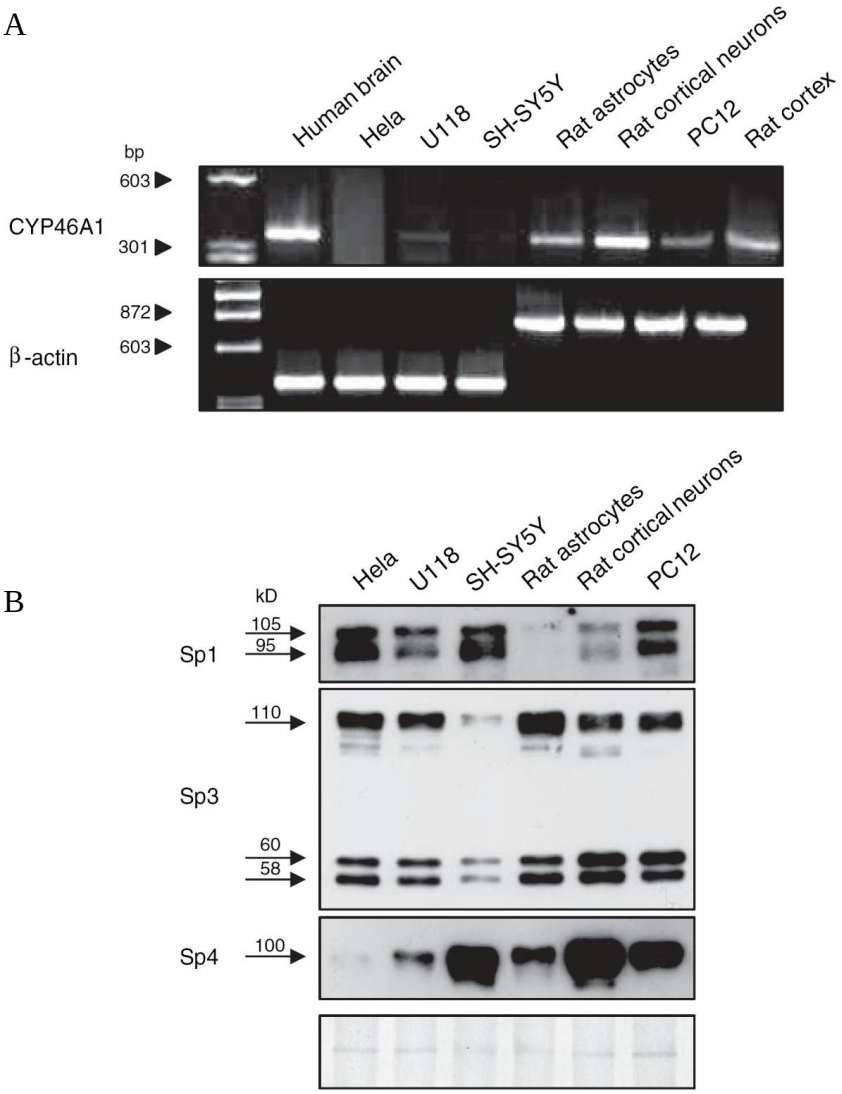

Figure 2.9. Correlation between CYP46A1 mRNA level with Sp family protein abundance in various cells types. (A) RT-PCR performed on total RNA isolated from HeLa, U118, SH-SY5Y, and PC12 cells, from primary cultures of rat cortical neurons and astrocytes, and from human brain and rat cortex. Reactions were performed in parallel under identical conditions using primers targeting the CYP46A1 mRNA, and the β-actin transcript as a control. Amplification products were separated in ethidium bromide- labeled agarose gel eletrophoresis. (B) Nuclear extracts were isolated from HeLa, U118, SH-SY5Y, and PC12 cells and from primary cultures of cortical neurons and astrocytes. Nuclear proteins (12.5 µg) were subjected to SDS-PAGE (8%), transferred to PVDF membranes, and the membranes were incubated with a specific anti-Sp1, -Sp3 and -Sp4 antibody. Protein loading control using Amido Black staining of the membrane is shown in the bottom panel. The results shown are representative of those obtained in three independent cultures.

2.5 Discussion

In this study, we have performed an analysis of the human *CYP46A1* promoter to identify DNA elements and transcription factors that regulate basal expression of this

gene. Deletion analysis revealed that a region spanning from -236 to -64 (+1 refers to the A of the first codon) is indispensable for basal expression of this TATA-less promoter activity. This GC-rich promoter region, which comprises consensus binding elements for Sp proteins and other zinc finger proteins as Egr-1 and Egr-2 transcription factors, is sufficient for high activity level in neuroblastoma cells.

Our data suggest that the neuronal-specific expression of CYP46A1 is regulated by positive regulatory elements rather than by a discrete repressive element, such as the neuronal restrictive silencer element (REST) that has been described to regulate cell type specificity of many neuronal genes (Ballas & Mandel 2005). Indeed, treatment of SH-SY5Y cells with mithramycin A, resulted in a significant reduction of promoter activity, suggesting a role of Sp family of transcription factors in CYP46A1 regulation. This chemotherapeutic drug has been used extensively as a tool to study Sp-dependent transcriptional responses, since it was shown to globally inhibit binding of Sp transcription factors to their cognate sites (Ray *et al.* 1989, Blume *et al.* 1991, Chatterjee *et al.* 2001).

Sp1 is generally considered a transcriptional activator, although an inhibitory region has recently been mapped in its extreme amino terminus (Bouwman & Philipsen 2002, Lee *et al.* 2005). Sp3 can function as an activator or a repressor depending on its isoform, PTMs and the sequence context of a given *cis*-element (Suske 1999, Bouwman & Philipsen 2002, Li *et al.* 2004, Sapetschnig *et al.* 2004). Sp3 has four isoforms that are the products of differential translational initiation (Sapetschnig *et al.* 2004). The short forms of Sp3 lack the subdomain A of the transactivation domain and act as repressors or weak activators. Although Sp3 is a transcription activator, it has also been described to reduce Sp1-dependent transcription of promoters containing adjacent Sp-binding sites by competing with Sp1 for promoter occupancy (Yu *et al.* 2003). Yet, there are promoters for which Sp3 activates rather than represses transcription (Gartel *et al.* 2000, Sowa *et al.* 1999). Despite the different roles of Sp1 and Sp3, they are generally thought to compete for the same binding sites. Previous studies have shown that both Sp1 and Sp3 are associated *in situ* with the GC boxes of a variety of promoters (Chan *et al.* 2004, Lee *et al.* 2004, Liu *et al.* 2004, Stoner *et al.* 2004, Zelko & Folz 2004, de Leon *et al.* 2005, Sun *et al.* 2005a).

In this study, we have shown that the *CYP46A1* promoter is highly transactivated by Sp1, Sp3 and Sp4. Moreover, we have identified a critical role of four repeated GC boxes in the regulation of *CYP46A1* promoter activity and in DNA– protein complex formation.

These GC-boxes were demonstrated to be both required and sufficient for high levels of promoter activity in neuroblastoma cells. Moreover, Sp1, Sp3, and Sp4 are able to bind to the four Sp-RE elements in the *CYP46A1* promoter. Combined mutation of Sp-RE-A and B, or Sp-RE-C and -D, reduced the promoter activity to approximately 30–40% of the wt, while only the mutation of the four Sp-RE sites, reduced activity to approximately 15% of the wt, and impaired transactivation by Sp proteins. Although we cannot rule out a role for other GC box-binding transcription factors, Sp3 and Sp4 were the major components of the protein–DNA complexes observed in extracts of primary cortical neurons, suggesting that these factors are essential for the activity of these GC boxes in neurons. Moreover, significant synergistic enhancement of the promoter transcriptional activation was obtained upon co-expressing Sp4 with Sp3.

Our studies suggest that Sp proteins contribute not only to control the level of *CYP46A1* expression but also to regulate the cell-type specificity. Since Sp1 and Sp3 are ubiquitously expressed, it may seem surprising that such transcription factors may contribute to the neuronal-specific expression of *CYP46A1*. Nevertheless, growing evidences suggest a role of Sp proteins in the cell-specific expression of several neuronal genes, namely cyclin-dependent kinase 5 (Ross *et al.* 2002b), N-methyl-D-aspartate receptor (Liu *et al.* 2004), secretin (Lee *et al.* 2004), superoxide dismutase 2 (Mao *et al.* 2006), neurogranin (Gui *et al.* 2006), and neurotrophin-3 (Ishimaru *et al.* 2007). Moreover, Sp proteins have been shown to be mediators of cyclooxygenase-2 induction by oxidative stress in primary neurons (Lee *et al.* 2006), and other results have suggested the need for Sp proteins as mediators of the transcriptional responses triggered by nerve growth factor (Yan & Ziff 1997, Billon *et al.* 1999, Sobue *et al.* 2005). Thus, the four Sp-RE boxes and their corresponding binding proteins may play an important role in regulating both the level of expression and cell-type specificity of the *CYP46A1* gene.

Our results suggest that CYP46A1 expression level will depend on the cell-specific differences in Sp proteins levels. We have found an inverse correlation between the Sp1/total Sp ratio and the CYP46A1 mRNA levels. Moreover, the ratios of Sp proteins present in the neuronal versus non-neuronal cells correlate well with their relative contributions to the Sp-RE binding activity. Indeed, these results are in agreement with our *in vitro* studies which show that the highest transactivation of the *CYP46A1* proximal promoter is achieved upon co-transfection of Sp3 and Sp4 expression vectors. Unfortunately, a good human cell model for the study of the human *CYP46A1* expression

has not yet been found, which prevented us to confirm Sp3 and Sp4 binding to the human promoter by chromatin immunoprecipitation assays.

It is interesting to notice that *CYP46A1* has been recently described to be expressed *in vivo* in neurons of the neural retina (Bretillon *et al.* 2007), cells where Sp4 is also expressed in high levels (Lerner *et al.* 2005). Nevertheless, we have not found any correlation between Sp4 levels and CYP46A1 mRNA in the continuous cell lines, which suggests that PTMs that may affect Sp protein activity, other transcription factors, or epigenetic modifications of the *CYP46A1* promoter, might also be important for basal expression of this gene. Indeed, posttranslational modifications might be affecting Sp1 and Sp3 gene regulation in the human neuroblastoma cell line, since PTMs, such as phosphorylation, glycosylation, ubiquitinylation, acetylation, ribosylation (Bouwman & Philipsen 2002), and SUMOylation (Spengler *et al.* 2005, Spengler & Brattain 2006) have been described to affect Sp protein function. However, little is known about Sp4 PTM that may affect its function.

Preliminary results from Ohyama and co-workers (Ohyama *et al.* 2006), revealed a potential for oxidative stress regulation of CYP46A1. Indeed, these authors have shown that when exposing SH-SY5Y cells to the organic hydroperoxide tert-butylhydroperoxide, the reporter promoter activity was stimulated ~2-fold. Interestingly, Sp1 and Sp3 have been described as neuronal oxidative stress-induced transcription factors (Ryu *et al.* 2003, Lee *et al.* 2006). Sp1 and Sp3 protein levels and function have also been described to be altered in neurodegenerative diseases that have been linked to oxidative stress and defects in cholesterol homeostasis, such as in Huntington's and Alzheimer's Disease (Dunah *et al.* 2002, Blalock *et al.* 2004, Qiu *et al.* 2006, Santpere *et al.* 2006). Moreover, Sp1 has been described to play an important role in regulating genes involved in the amyloid cascade (BACE1, BACE2, Tau) (Sun *et al.* 2005b, Heicklen-Klein & Ginzburg 2000, Christensen *et al.* 2004).

Of interest is the observation that many LXR target genes, involved in lipid metabolism and reverse cholesterol transport, present functional Sp binding elements in the promoter. The combination of LXREs and Sp sites has been identified and characterized on the promoter of the cholesterol ester transfer protein gene (Le Goff *et al.* 2003, Luo & Tall 2000), ABCA1 (Thymiakou *et al.* 2007), apoE (Zannis *et al.* 2001), and lipoprotein lipase (Yang & Deeb 1998, Zhang *et al.* 2001). Thus, the cross-talk between LXR activation and members of the Sp family of transcription factors seems to play an important role in the modulation of lipid metabolism.

In conclusion, we have demonstrated the importance of Sp family of transcription factors as major regulators of *CYP46A1* basal expression. Although, all Sp proteins are transactivators of the *CYP46A1* promoter, neuronal differentiation may differentially modify the interaction and function between individual Sp factors and the *cis*-elements herein characterized, and, thus set up the brain-specific expression of the *CYP46A1* gene. These findings may imply that cell-type specificity of transcription factors in the CNS may include a unique aspect of neurons: the substitution of Sp1 by Sp4.

Since, 24OHC has been described as exerting an unique modulatory effect on amyloid precursor protein processing by increasing the alpha-secretase activity as well as the α- / β-secretase activity ratio (Famer *et al.* 2007), it is consequently possible that transcriptional regulators such as Sp proteins may serve as modifiers in neurodegenerative pathogenesis.

Therefore, the identification of *CYP46A1* as a target gene of Sp proteins can help to elucidate the molecular events involved in the regulation of gene expression in the central nervous system, but also in processes associated with neurodegenerative disorders.

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NEURONAL DIFFERENTIATION ALTERS THE RATIO OF SP TRANSCRIPTION FACTORS RECRUITED TO THE *CYP46A1* PROMOTER

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3.1 Abstract

CYP46A1 is a neuron-specific cytochrome P450 that plays a pivotal role in maintaining cholesterol homeostasis in the CNS. However, despite its importance, the molecular mechanisms underlying human *CYP46A1* expression are still poorly understood, partly due to the lack of a cellular model that expresses high levels of CYP46A1 mRNA. Our previous studies demonstrated that Sp transcription factors control the level of *CYP46A1* expression, and are most probably responsible for cell type specificity. Herein, we have differentiated Ntera2/clone D1 (NT2) cells into post-mitotic neurons, and identified for the first time a human cell model that expresses high levels of CYP46A1 mRNA. Immunocytochemistry and western blot analysis revealed a decrease in the ubiquitous Sp1 protein levels, concomitant with the increase in CYP46A1 mRNA levels. This decrease was correlated with changes in the ratio of Sp proteins associated to the *CYP46A1* proximal promoter in the context of native chromatin. Indeed, by ChIP analysis a 50% decrease in the Sp1 protein recruited to the proximal promoter of this gene was detected. To examine if the increase in the (Sp3+Sp4)/ Sp1 ratio was observed at the level of other Sp-regulated neuronal promoters, we have selected four genes known to be expressed in the human brain, MOR, reelin, GRIN1 and GRIN2A, and analyzed the Sp1, Sp3 and Sp4 binding pattern to these promoters, in undifferentiated NT2 and in post-mitotic neurons. ChIP data pointed to the fact that, contrary to what we initially hypothesize, the dissociation of Sp1 from promoter regions is not a common feature amongst neuronal Sp-regulated genes, since we could not identify any conserved patterns in the recruitment of Sp. Our results, suggest that binding of Sp proteins to DNA in a neuronal chromatin environment is highly dependent on promoter context, implying that although the Sp1 levels are significantly decreased after neuronal differentiation, Sp1-dependent transactivation of specific neuronal promoter is most likely to occur.

3.2 Introduction

Cholesterol metabolism in the central nervous system (CNS) is distinct from that in other tissues. No uptake of cholesterol from the plasma to the brain occurs, as the blood brain barrier (BBB) prevents the diffusion of large molecules at the tight junctions of brain capillary endothelial cells (Bjorkhem & Meaney 2004). Thus, virtually all the cholesterol necessary to the good functioning of the CNS is synthesized *in situ* (Dietschy & Turley 2004), and is efficiently recycled. To maintain cholesterol homeostasis the brain relies on a unique catabolism pathway, the conversion of cholesterol into 24(*S*)-hydroxycholesterol (24OHC) (Bjorkhem & Meaney 2004), which is thought to be the most important mechanism of cholesterol turnover (Bjorkhem *et al.* 1997). The enzyme responsible for the conversion of cholesterol into 24OHC was found to be the neuronal specific cytochrome P450 46A1 (CYP46A1) (Lund *et al.* 1999).

The *CYP46A1* gene is localized in chromosome 14q32.1, encompasses 15 exons and 14 introns and encodes the cholesterol 24-hydroxylase (Lund *et al.* 1999). Unlike other cytochrome P450 sterol hydroxylases that are predominantly expressed in the liver (Xie *et al.* 2003), CYP46A1 is highly expressed in neurons of several brain regions and also in neurons of the neural retina (Lund *et al.* 1999, Bretillon *et al.* 2007).

Despite the pivotal role of CYP46A1 in the maintenance of brain cholesterol homeostasis, only recently the structural and functional characterization of the human *CYP46A1* 5' flanking region was described (Ohyama *et al.* 2006, Milagre *et al.* 2008). Unlike what is common in other P450 genes, there is no substrate-dependent transcriptional regulation of *CYP46A1* expression, and treatment of SH-SY5Y neuroblastoma cells with a broad spectrum of endogenous and exogenous compounds also did not result in any significant change in *CYP46A1* promoter activity (Ohyama *et al.* 2006). In our previous studies we have mapped a promoter region indispensable for basal expression of this gene that responds to the ubiquitous specificity protein (Sp)1 and Sp3 transcription factors, as well as the brain-enriched Sp4. Our results suggested that the cell-type specific expression of Sp transcription factors, namely the substitution of Sp1 by Sp4 in neurons, is responsible for the basal expression of the *CYP46A1* gene (Milagre *et al.* 2008). Moreover, we have also demonstrated the importance of these transcription factors in the *CYP46A1* response to the epigenetic modifiers trichostatin A and 5'-Aza-2'-deoxycytidine (Nunes *et al.* 2010, Milagre *et al.* 2010), reinforcing the importance of the different Sp transcription factors in the regulation of *CYP46A1*.

Comparison of the human and rat *CYP46A1* promoter sequences revealed significant differences, upstream of the core promoter (Milagre *et al.* 2008), suggesting that results from studies in rat primary cortical neurons may not reflect what happens in human brain. Therefore the lack of a cellular model that expresses high levels of human CYP46A1 mRNA has limited the insight into the molecular mechanisms underlying the expression of this gene, namely the validation of our initial hypothesis that a shift in the ratio between Sp proteins recruited to the *CYP46A1* promoter, favouring the neuronal enriched Sp4, would be responsible for an increase in *CYP46A1* expression. Interestingly, Steve Barger has also put forward the hypothesis that the substitution of the ubiquitous Sp1 by Sp4 in neuronal cells, would be responsible for cell-type specificity expression in the CNS (Mao *et al.* 2006).

Herein we aimed to confirm if an alteration in the ratio of Sp proteins recruited to the *CYP46A1* promoter in human post-mitotic neurons, would be responsible for the neuronal-specific expression of this gene, and to analyze if such a switch, with important implications on the control of neuronal gene expression, also occurs in the promoters of other Sp-regulated genes, expressed in neurons. We have used the Ntera2/clone D1 (NT2) human teratocarcinoma cell line that can be induced by retinoic acid to differentiate into human postmitotic neurons (NT2N) and other cell types of neuronal lineage (Guillemain *et al.* 2000), and demonstrated for the first time a significant increase in CYP46A1 mRNA levels that was concomitant with the decrease in the levels of Sp1 associated with the proximal promoter of this gene. Nevertheless, we did not identify any conserved alteration in the Sp protein binding patterns to the other neuronal Sp-regulated gene promoters analyzed, suggesting that Sp transcription factors DNA binding and transcriptional activity is highly dependent on the neuronal chromatin context, reinforcing the knowledge that Sp transcription factors act as part of complex networks in neuronal gene regulation.

3.3 Methods

3.3.1 Cell Culture

NT2 testicular embryonal carcinoma cells were cultured as previously described (Cheung *et al.* 1999, Megiorni *et al.* 2005), with minor modifications. Briefly, NT2 cells were maintained in high glucose DMEM (Sigma Aldrich Inc., St Louis, MO, USA)

supplemented with 10 % heat inactivated fetal bovine serum (GIBCO, Rockville, MD, USA), 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (Sigma), at 37 °C in a humidified 5 % CO_2 atmosphere. To obtain NT2 neurons (NT2N), cells were seeded at $1x10^6$ cells/ml in 100 mm bacterial grade Petri-dish to initiate cell aggregation and 10 µM *all-trans* retinoic acid (RA) (Sigma) was added in the following day. Medium was changed every 3 days and cells were maintained in RA-containing medium for 3 weeks. Afterwards, the cells were seeded in poly-D-lysine and Matrigel® (PDL-MG) coated tissue culture grade Petri dishes and maintained in high glucose DMEM supplemented with 5% FBS (Hyclone, Logan, UT, USA), 100 units/ml penicillin, 100 µg/ml streptomycin and mitotic inhibitors [1 µM cytosine D-arabinofuranoside, 10 µM fluorodeoxyuridine and 10 µM uridine (all from Sigma)].

3.3.2 Antibodies

The antibodies used in western blot, immunocytochemistry (ICC) and chromatin immunoprecipitation (ChIP) are listed in Table 3.1.

Table 3.1 – List of the antibodies used in western blot, immunocitochemistry and chromatin immunoprecipitation (ChIP).

Protein	Clone/ Reference	Source	Assay
Sp1	PEP-2	Santa Cruz Biotechnology	Western Blot, ICC, ChIP
Sp3	D-20	Santa Cruz Biotechnology	Western Blot, ICC, ChIP
Sp4	V-40	Santa Cruz Biotechnology	Western Blot, ICC, ChIP
TUJ-1	MMS-435P	Covance	ICC
Nestin	AB5922	Millipore	ICC
MAP2	MAB3418	Millipore	ICC
NF200	N4142	Sigma	ICC

3.3.3 Immunocytochemistry

Cells were washed with ice-cold PBS, fixed in 4 % paraformaldehyde / PBS, pH 7.4, permeabilized with 0.1 % Triton X-100® in PBS, and then treated with blocking solution (10 % FBS, 0.05 % Triton X-100® in PBS). Incubation with specific primary antibodies was performed overnight at 4ºC in a humidified chamber, followed by incubation with

FITC-conjugated goat anti-rabbit IgG antibody (Sigma) for 1 hour at room temperature. Finally, cells were rinsed with PBS, mounted in fluorescent mounting medium containing 5 µg/ml Höechst dye 33258 (Sigma). Fluorescence was visualized using a Leica DC 100 camera (Leica, Wetzlar, Germany) adapted to an Axioskop® microscope (Zeiss, Göttingen, Germany) and photographed by using Image Manager 50 software (Leica). Control experiments for non-specific binding were performed in parallel by omission of the primary antibody.

3.3.4 Expression Analysis

Total cell RNA was extracted using Trizol Reagent (Invitrogen Carlsbad, CA, USA) following manufacturer's instructions. Real-Time PCR analysis for CYP46A1 mRNA levels was performed as previously described (Nunes *et al.* 2010). Real-Time PCR analysis for reelin, GRIN1, GRIN2A and MOR was performed using SYBR green Master Mix in an ABI 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA) and specific primers (Table 2) for reelin, GRIN1, GRIN2A and MOR. Results presented are from three individual experiments and each sample was assayed in triplicate. mRNA levels were normalized to the level of β-actin, and are presented as fold change from controls, using the $^{\Delta\Delta}$Ct method (CYP46A1, reelin, MOR and GRIN2A) or using the standard curve method and expressed as fg of target mRNA per ng of β-actin mRNA (GRIN1). Six candidate genes (β-actin, GAPDH, 18s, ATPb5, UBC, eIF4A2) were analyzed with the Norm Finder algorithm (Andersen *et al.* 2004) and β-actin was found to be the most stable single gene to be used as an endogenous control, in the experimental conditions used.

3.3.5 Western blot analysis

Cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton-X 100) containing 1 mM dithiothreitol and a protease inhibitor mixture (Roche Diagnostics GmbH, Penzberg, Germany). After incubation at 4 ºC, for 30 minutes, samples were sonicated four times for 4 seconds each, on ice, followed by centrifugation at 14,000 rpm for 30 min. Proteins were subject to 7.5% SDS-PAGE gels and electroblotted onto Immobilon P (Millipore, Bedford, MA, USA). After visualization of the transferred proteins by amido black staining, the membranes were incubated with

specific antibodies. Results were quantified using the Quantity One version densitometry analysis software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

3.3.6 Chromatin immunoprecipitation

NT2 cells (1 x 10^7) were fixed with 1% formaldehyde for 10 min at room temperature. After cross-linking, the reaction was quenched with 0.125 M of glycine for 10 min at room temperature. Cells were washed twice with ice-cold PBS, pelleted by centrifugation, resuspended in 1 ml of cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% Igepal and 1 x protease inhibitor cocktail), and incubated 30 min at 4ºC. After centrifugation the nuclei were resuspended in nuclei lysis buffer (50 mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS and 1 x protease inhibitor cocktail) and incubated for 10 min on ice. The soluble chromatin with a size range of 0.5 kb to 0.9kb was prepared by sonication. After centrifugation to remove cell debris, chromatin was pre-cleared [1 h at 4ºC with a 50% gel slurry of protein A/G – agarose beads saturated with salmon sperm DNA and bovine serum albumin (Upstate)], diluted in IP dilution buffer (0.01% SDS, 0.5% Triton X-100, 2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 100 mM NaCl and 1 x protease inhibitor cocktail), and 10% of the supernatant was used as input. The diluted chromatin was incubated overnight at 4°C with the antibodies of interest and the immune complexes were recovered by 1 h incubation at 4ºC with a 50% gel slurry of protein A/G – agarose beads (Upstate). The precipitated complexes were washed sequentially with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl and 1 x protease inhibitor cocktail), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl and 1 x protease inhibitor cocktail), LiCl buffer (1 mM EDTA, 10 mM Tris-HCl pH 8.1, 250 mM LiCl, 1% Igepal, 1% deoxycholic acid and 1 x protease inhibitor cocktail) and twice with Tris – EDTA buffer (1 mM EDTA and 20 mM Tris-HCl pH 8.1), and extracted twice with freshly prepared elution buffer (100 mM $NaHCO_3$ and 1% SDS) with mild vortexing. The cross-linking between DNA and proteins was reversed by incubation with 0.3 M NaCl, overnight at 67ºC, in the presence of RNase A. Samples were then digested with proteinase K at 45ºC for 1 h. DNA was purified using QIAquick PCR purification kit (Qiagen). The recovered DNA was analyzed by real-time PCR with SYBR green Master Mix in an ABI 7300 sequence detection system (Applied Biosystems). The real-time PCRs were performed using primers that covered *CYP46A1* proximal promoter region as

previously described (Nunes *et al.* 2010), or primers for the proximal promoter regions of reelin, GRIN1, GRIN2A and MOR (Table 3.2).

Table 3.2 – List of primers used in qPCR and qChIP

Gene	Primer name / TaqMan Assay	Sequence (5'→ 3') / Source	q PCR	RT-PCR	Ch IP	Reference
CYP46A1	CYP46A1 HS00198510_M1	Applied Biosystems	x			
	qChIP+1rCYP46f	5' gcggacctgagtctgaagag 3'				(Nunes *et al.* 2010)
	qChIP+1rCYP46f	5' aatcacaactccgcttctgg 3'			x	
β Actin	ACTB HS99999_M1	Applied Biosystems	x			
	qPCRHuACTINf	5'-ctggaacggtgaaggtgaca-3'				
	qPCRHuACTINr	5'-aagggacttcctgtaacaatcca-3'	x			
	hACTINf	5'-gcaccacaccttctacaatgagc-3'				(Milagre *et al.* 2008)
	hACTINr	5'-aatgtcacgcacgatttcccgc-3'		x		
neuroD	hNeuroDFwd	5'-gccccagggttatgagactatcact-3'	x			(Megiorni *et al.* 2005)
	hNeuroDRev	5'-ccgacagagcccagatgtagttctt-3'				
reelin	qPCRReelinFwd	5'-gcaccagccaaaggacttca-3'	x			(Haas *et al.* 2002)
	qPCRReelinRev	5'-gttgccaccagcgcagtaa-3'				
	qCHIPReelinFwd	5'- ccgggacacgtgtggcggcg-3'				(Kundakovic *et al.* 2009)
	qCHIPReelinRev	5'-ggcgagaagaagggcggacggg-3'			x	
GRIN1	qPCRGRIN1Fwd	5'-ccaggcggagagacagagaa-3'	x			(Bullock *et al.* 2008)
	qPCRGRIN1Rev	5'-ctccttgcatgtcccatcact-3'				
	qCHIPGRIN1Fwd	5'-gagcttcagccatccttcaa-3'				(Stadler *et al.* 2005)
	qCHIPGRIN1Rev	5'-ccttttccagagccttaccc-3'			x	
GRIN2A	qPCRGRIN2Afwd	5'-atgggaaaaggtgggcaagt-3'	x			(Bullock *et al.* 2008)
	qPCRGRIN2ARev	5'- cctccagggtgacgatgct-3'				
	qCHIPGRIN2AFwd	5'-gctcctcccttgaccatgta-3'				(Stadler *et al.* 2005)
	qCHIPGRIN2ARev	5'-accagaagaggtgagccaga-3'			x	
MOR	qPCRMORFwd	5'-ctgggtcaacttgtcccact-3'	x			(Bedini *et al.* 2010)
	qPCRMORRev	5'-tggagtagagggccatgatc-3'				
	qCHIPMORFwd	5'-actccttggatcgctttgc-3'				(Liu *et al.* 2009)
	qCHIPMORRev	5'-cctcccaccttagtagttcaca-3'			x	

3.3.7 Statistical Analysis

Statistical analysis was performed using the Student's t-test, Kruskal-Wallis test with Dunn's post-hoc test and the ANOVA one-way test with the Tukey HSD post-hoc test or the Tukey HSD for unequal N (Spjotvoll/Stoline test). All analysis was performed using the STATISTICA (data analysis software system), version 9.1 StatSoft, Inc. (2010) or Prism 5.0 software (GraphPad Software).

3.4 Results

3.4.1 Differentiation of the NT2 cell line

The first protocols for neuronal differentiation of NT2 cells were very time consuming and laborious taking up to 2 months before NT2 neurons could be obtained (Andrews 1984, Pleasure *et al.* 1992). Therefore in the last decade there has been an effort to develop a differentiation culture system with reduced differentiation time. Indeed, cell aggregate methods for NT2 neuronal differentiation that rely on the use of a high concentration of RA, together with the influence of cell-cell adhesion in freely floating aggregates have been described (Megiorni *et al.* 2005, Paquet-Durand *et al.* 2003, Horrocks *et al.* 2003).

The differentiation process is divided into two stages: the differentiation stage, in which NT2 cells are treated with RA for 21 days, and the replate stage, in which the NT2 cells are replated in PDL-MG coated tissue culture grade petri dishes and treated with mitotic inhibitors for 1 week; herein we have named the days of RA treatment NT2 d0, d1 and so forth until d21, and the 7^{th} day after replating as NT2N R7d.

In order to assess if RA treatment was inducing the differentiation of the aggregate culture, we tested the expression of the basic helix-loop-helix transcription factor involved in neuronal differentiation (NeuroD) by RT-PCR in the first days of differentiation (Figure 3.1 A). Our results show an increase in NeuroD mRNA levels two days after addition of RA, which was in accordance with previous reports (Jain *et al.* 2007, Megiorni *et al.* 2005, Przyborski *et al.* 2003).

To confirm neuronal differentiation we assessed the presence of cytoskeletal markers both in immature precursors and mature neurons by immunofluorescence (Figure 3.1 B). NT2 d0 precursor cells express the intermediate filament nestin, a typical marker for

neuronal precursors (Figure 3.1 B), while displaying only faint immunoreactivity to the antibody against type III β-tubulin (TUJ-1). The replate in PDL-MG coated surfaces and the incubation with mitotic inhibitors, in the absence of RA, led to the decrease of proliferating NT2 precursor cells. Indeed, NT2N R7d neurons already displayed immunoreactivity for microtubule-associated protein 2 (MAP-2), neurofilament 200 (NF200), and TUJ-1, and extensive neuritic networks. Counterstaining with Höechst dye revealed the presence of a few epitheloid cells (large nuclei) that do not have positive staining for the neuronal markers (Figure 3.1 B).

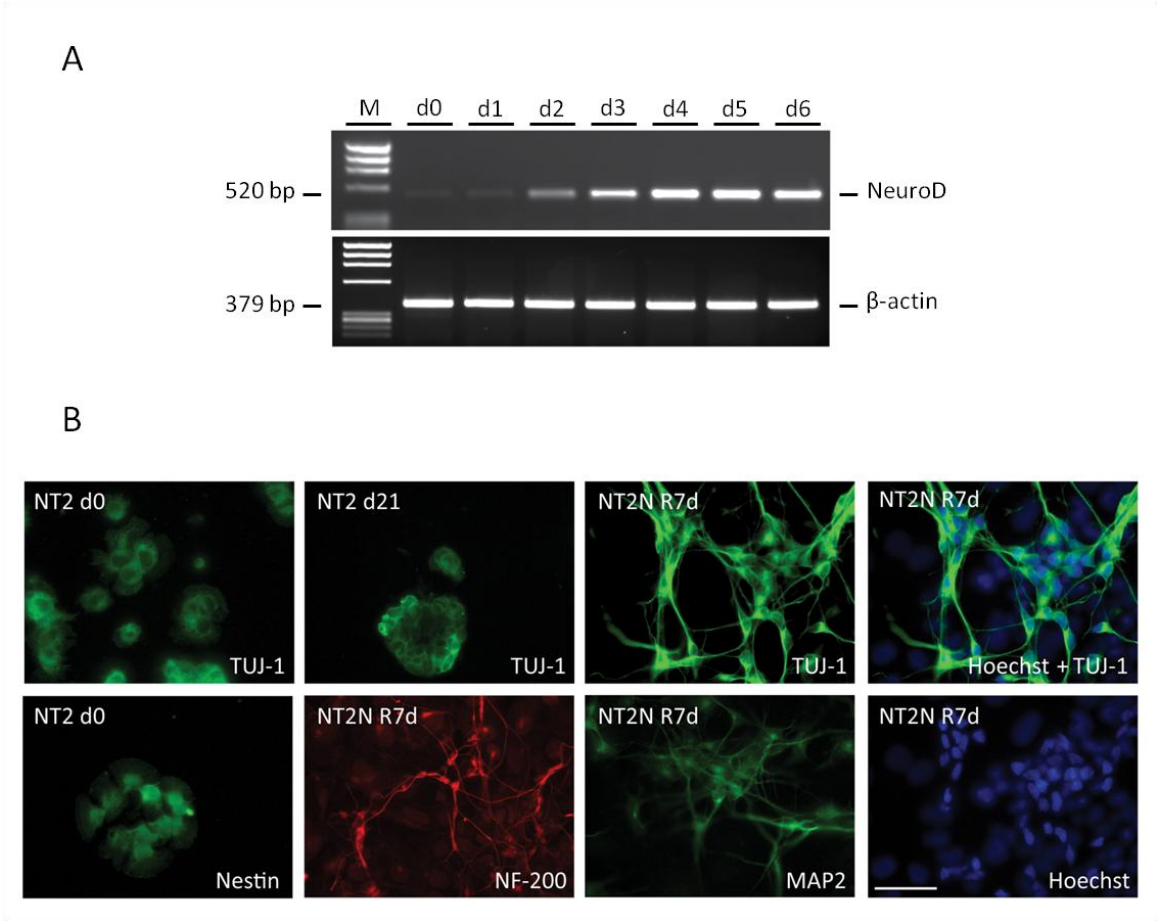

Figure 3.1. Analysis of neuronal markers during differentiation of NT2 cells. NT2 cells were treated with retinoic acid (RA) for 21 days. The cells were then replated on culture plates coated with PDL-MG and maintained for 1 week in the absence of RA and in the presence of mitosis inhibitors (NT2N). A) NeuroD mRNA levels, during NT2 differentiation, were determined by RT-PCR. β-actin was used as a control. B) Immunodetection of the neuronal markers TUJ-1, MAP2, Nestin and NF200. The results shown are representative of those obtained in at least three independent cultures. *Scale bar = 100 µm.*

3.4.2 CYP46A1 mRNA levels during NT2 neuronal differentiation

After having confirmed that NT2N cells express several markers of post-mitotic neurons, we determined the CYP46A1 mRNA levels throughout differentiation (Figure 3.2). We observed a 10.4 fold increase in CYP46A1 mRNA levels at NT2N R7d, when compared with the NT2 d0 cells (ANOVA one-way test: $F = 5.4$, $df = 5$, $*p < 0.001$), demonstrating the up-regulation of this gene after neuronal differentiation.

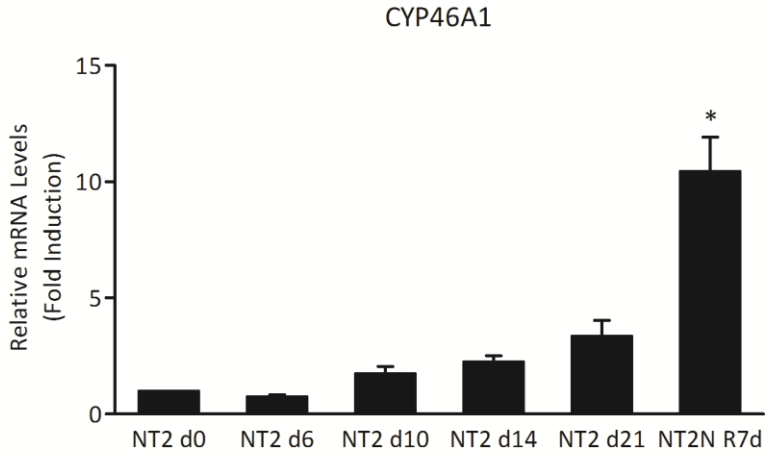

Figure 3.2. CYP46A1 mRNA expression analysis during differentiation of NT2 cells. Real-time PCR analysis of steady-state mRNA transcript levels in NT2 cells during differentiation. mRNA levels were normalized to the level of β-actin, expressed as fold change relative to undifferentiated cells and represent means ± SEM from at least three individual experiments ($*p < 0.001$).

3.4.3 Expression pattern of other Sp regulated genes in undifferentiated and differentiated NT2 cells

Afterwards we have also evaluated the mRNA levels of other genes expressed in neuronal cells and previously described has being regulated by Sp proteins, in undifferentiated (NT2 d0) and in differentiated NT2 (NT2N R7d) cells. We have chosen the μ opioid receptor gene (MOR) that has been shown to be highly regulated at the level of transcription (Xu & Carr 2001), reelin that codes for a serine protease involved in synaptic plasticity (Chen *et al.* 2005), and GRIN1 and GRIN2A that code for N-methyl-D-aspartate (NMDA) receptors subunits, essential for synaptic plasticity (Harris *et al.* 1984) (Figure 3.3). We observed low mRNA levels of reelin and MOR in undifferentiated cells, but these significantly increased in NT2N cells, by 36.7- and 6.5- fold (Student's *t*-

test, †p < 0.01 and §p < 0.05), respectively. Furthermore, GRIN1 mRNA was not detected in undifferentiated cells whereas in NT2N R7d it was readily detected. In contrast, we did not observe any significant variation on the mRNA levels of GRIN2A. These data demonstrate the up-regulation of genes, other than *CYP46A1*, that have been described as targets of Sp proteins, during differentiation of NT2 cells into human neurons.

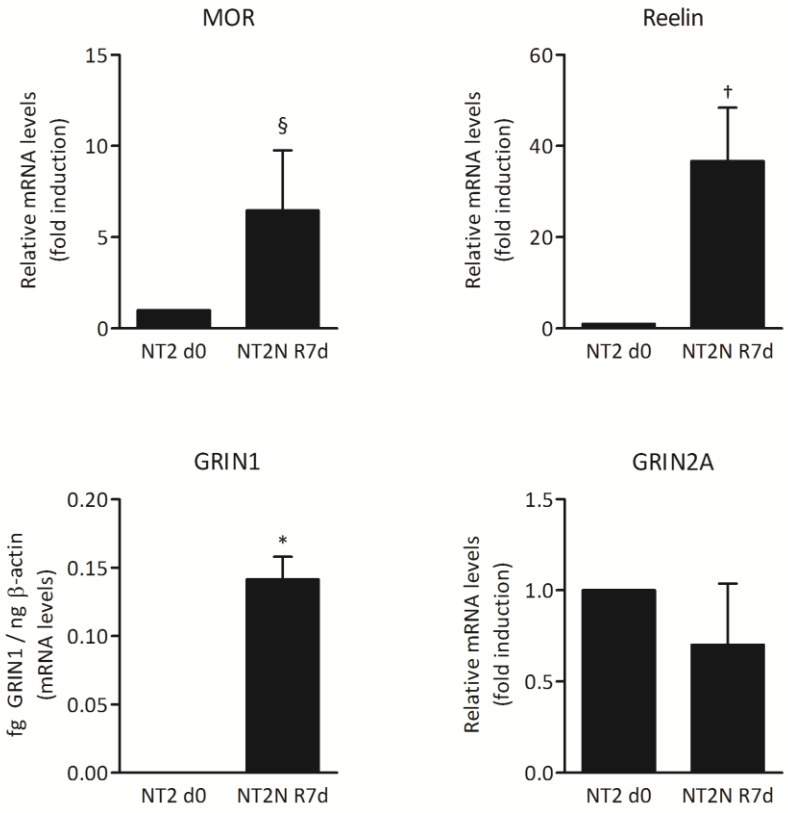

Figure 3.3. mRNA expression analysis of MOR, reelin, GRIN1 and GRIN2A in NT2 d0 and NT2N R7d cells. Real-time PCR analysis of steady-state mRNA transcript levels in undifferentiated and differentiated NT2 cells. Values were normalized to the internal standard β-actin and expressed as fg of target mRNA per ng of β-actin mRNA (GRIN1) or as fold change relative to undifferentiated cells (reelin, MOR and GRIN2A) and represent means ± SEM from at least three individual experiments (§p < 0.05; †p < 0.01; *p < 0.001).

3.4.4 Sp transcription factors levels during differentiation of NT2 cells into neurons

We evaluated by immunocytochemistry and western blot if the protein levels of the different Sp transcription factors were altered during differentiation of NT2 cells (Figure 3.4), since Sp1 and Sp3 are described as being expressed in all mammalian cell types, while Sp4 expression is mainly restricted to the brain (Suske 1999).

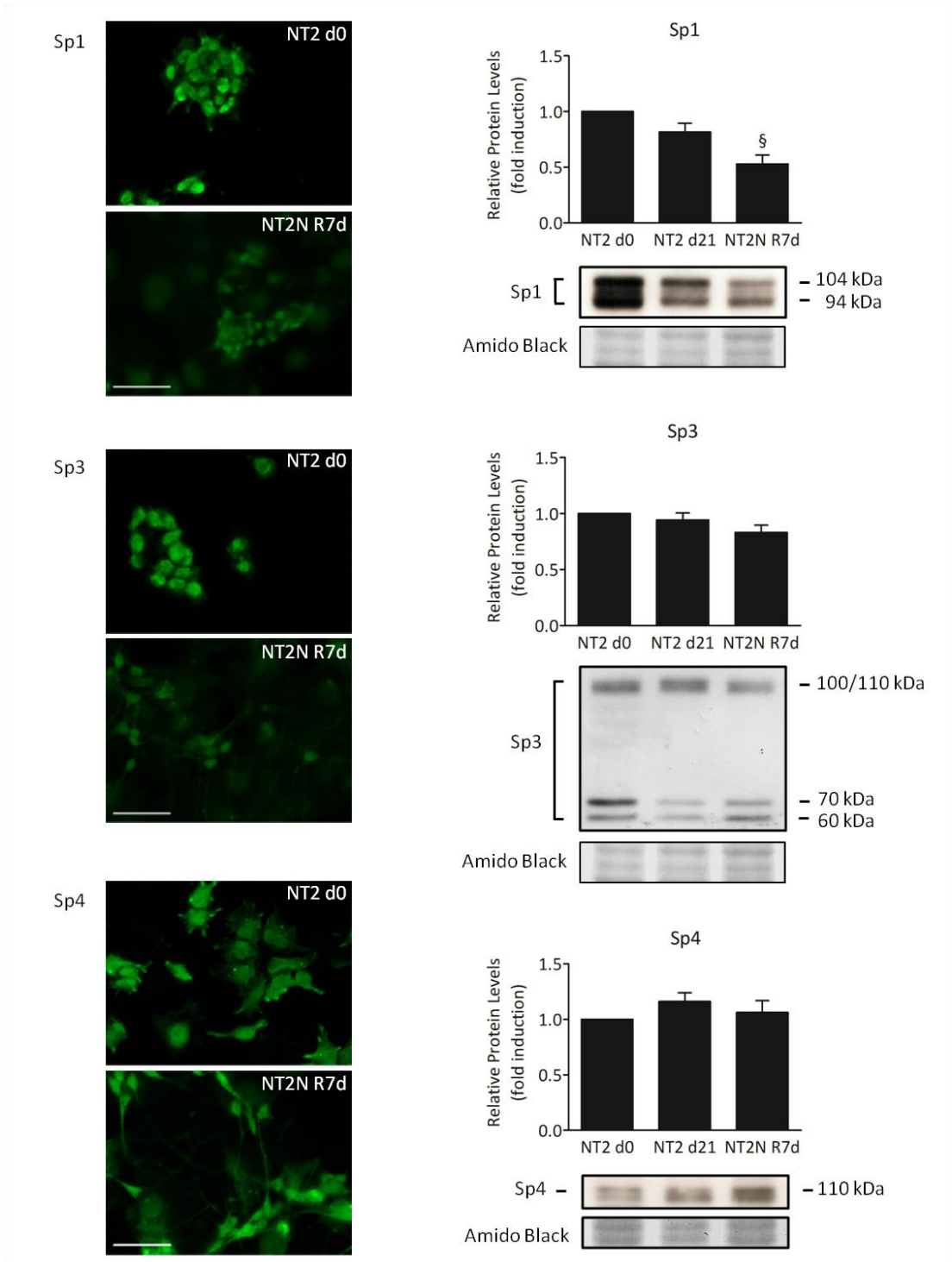

Figure 3.4. Sp protein levels during differentiation of NT2 cells. A) Immunodetection of the Sp transcription factors Sp1, Sp3 and Sp4 in NT2 d0 and NT2N R7d cells. B) Western blot analysis of the Sp proteins levels during NT2 neuronal differentiation. Nuclear extracts were subjected to SDS-PAGE, transferred to PVDF membranes and the membranes were incubated with specific anti -Sp1, -Sp3 and -Sp4 antibodies. Protein loading control using Amido Black staining of the membrane is shown in the bottom panel. The results shown are representative of those obtained in three independent cultures. Data represent means ± SEM and are expressed as fold change relative to control cells (§$p < 0.05$). *Scale bar = 100 µm.*

Immunocytochemistry demonstrated that although all the Sp transcription factors were present in both the undifferentiated NT2 d0 and the differentiated NT2N R7d cells, Sp1 and Sp3 levels seemed to slightly decrease. Since it is difficult to quantitatively analyze immunostaining in cells with marked morphological differences such as those seen in the undifferentiated and differentiated cells, we further evaluated the different Sp protein levels by western blot analysis. We confirmed a decrease in protein levels of ubiquitous Sp1 during the differentiation of NT2 cells into neurons. In fact, Sp1 decreases to about 50% in NT2N R7d cells (Kruskal-Wallis test, §$p < 0.05$), when compared with undifferentiated NT2 d0. However, we did not observe any significant change in Sp3 levels throughout differentiation, nor in the levels of the brain enriched Sp4. These results show that there is a shift in the ratio of Sp1/Sp3/Sp4 nuclear levels after neuronal differentiation of NT2 cells.

3.4.5 Recruitment of Sp transcription factors to the GC-rich proximal promoters of *CYP46A1* and other Sp-regulated genes in NT2 d0 and NT2N R7d cells

To further understand if the decrease in Sp1 protein levels observed in NT2N cells lead to a modification in the multiprotein complex bound to the *CYP46A1* promoter, we performed ChIP assays. We detected Sp1, Sp3 and Sp4 associated to the *CYP46A1* proximal promoter region (Figure 3.5), both in NT2 d0 and in NT2N R7d cells. Moreover, we observed a significant decrease in the binding of the Sp1 transcription factor to the proximal promoter of *CYP46A1*, after neuronal differentiation (Student's *t*-test, §$p < 0.05$), whereas the levels of both Sp3 and Sp4 association remained unchanged. These data confirmed our previous results, showing that Sp proteins are recruited to the *CYP46A1* proximal promoter in human cells that express high levels of CYP46A1 mRNA. Furthermore, these results also indicate that there is a shift in the ratio of the Sp1/Sp3/Sp4 transcription factors bound to the *CYP46A1* proximal promoter region in the differentiated cells, which is in agreement with our previous hypothesis (Milagre *et al.* 2008).

Subsequently, we performed ChIP assays to determine the association of Sp proteins to the proximal promoters of the reelin, MOR, GRIN1 and GRIN2A genes, before and after NT2 differentiation. Our results showed that both Sp1 and Sp3 were present in the region spanning from -220 to +70 of the reelin promoter in undifferentiated cells (Student's *t*-test, †$p < 0.01$ and §$p < 0.05$, respectively), whereas Sp1 was significantly

dissociated from the promoter region in NT2N R7d cells, since there were no longer significant differences between the chromatin recovered with the anti-Sp1 antibody, and that recovered with IgG. Moreover, we observed that Sp4 was recruited to the reelin promoter (Student's *t*-test, §$p < 0.05$) in post-mitotic neurons. In contrast, the anti-Sp1 antibody was the only capable of immunoprecipitating the region spanning from -346 to -227 of the MOR promoter (Student's *t*-test, †$p < 0.01$) and only in NT2N cells. By analyzing the recruitment of Sp proteins to the GRIN1 promoter, we could detect all of the studied Sp transcription factors associated with the -89 to +19 region of this gene in the undifferentiated cells (Student's *t*-test, *$p < 0.001$ for Sp1 and Sp3 and †$p < 0.01$ for Sp4), whereas after differentiation the levels of the Sp1 and Sp3 bound to the promoter significantly decreased (Student's *t*-test, †$p < 0.01$ and §$p < 0.05$, respectively), and Sp4 could no longer be detected.

Lastly, we have also analyzed recruitment of Sp factors to the GRIN2A promoter, although we did not detect and increase in GRIN2A mRNA in NT2N cells. Interestingly, we could also observe an alteration in the Sp proteins associated with the -222/ -118 region. Our results revealed that neuronal differentiation did not induce an alteration in the binding of Sp1 to the GRIN2A promoter region, while association Sp3 was detected only in the NT2N R7d cells (Student's *t*-test, §$p < 0.05$). Sp4 was never found to be associated with GRIN2A promoter. Our data demonstrate that the replacement of Sp1 by Sp4 after neuronal differentiation is not a common feature amongst the studied promoters, highlighting the involvement of other regulatory mechanisms.

3.5 Discussion

Cholesterol 24-hydroxylase is a neuron specific cytochrome P450 that plays a pivotal role in the maintenance of brain cholesterol homeostasis. Although increasing evidence suggests that upregulation of CYP46A1 may be a therapeutic strategy in Alzheimer's Disease treatment (Hudry *et al.* 2009), due to the lack of an appropriate cell model system that expresses high levels of human *CYP46A1*, the unraveling of the regulatory mechanisms governing its neuronal specific expression has been impaired.

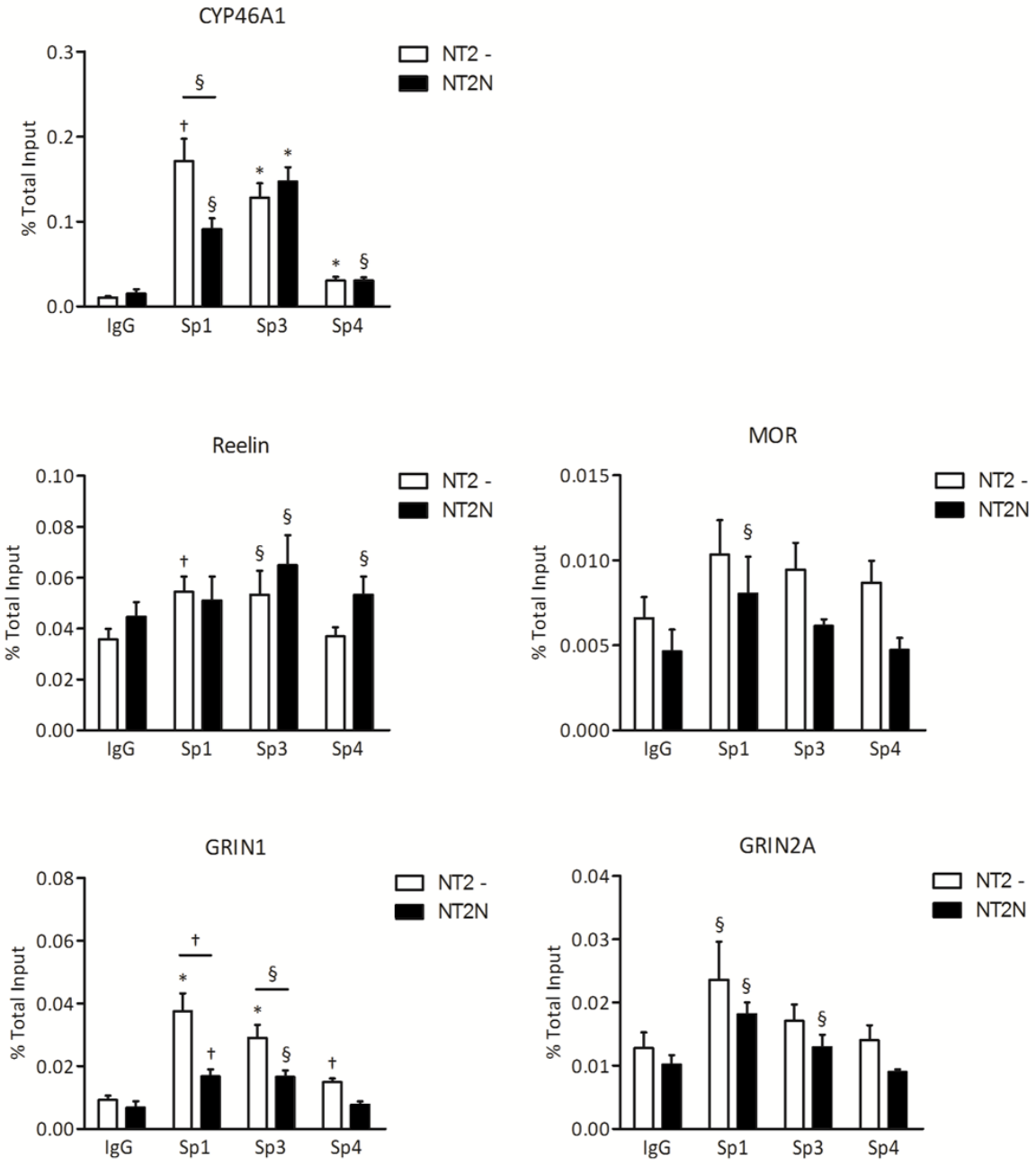

Figure 3.5. Recruitment of Sp transcription factors to the proximal promoter of CYP46A1 and other Sp-regulated genes in NT2 and NT2N cells. Recruitment of Sp transcription factors to the proximal promoter of CYP46A1, reelin, MOR, GRIN1 and GRIN2A. Chromatin from NT2 d0 and NT2N R7d was prepared and precipitated with antibodies directed against -Sp1, -Sp3, -Sp4. After DNA recovery, the precipitates were evaluated by real-time PCR. Results are expressed as fold change over IgG and represent means of at least three independent experiments ± SEM (§$p < 0.05$; †$p < 0.01$; *$p <$ 0.001).

To overcome this problem, herein we have induced neuronal differentiation of human NT2 cells. Post-mitotic NT2 neurons have been widely characterized and numerous studies have shown that, after RA treatment, these cells express several neuronal markers such as MAP1 and 2, Tau, synaptophysin and chromogranin (Pleasure *et al.* 1992), as well as neuropeptides, neurotrophic factors, neurotransmitters and neurotransmitter receptors (Guillemain *et al.* 2000, Llanes *et al.* 1995, Zigova *et al.* 2000, Sodja *et al.* 2002). Herein we show that CYP46A1 mRNA levels are significantly increased after differentiation of NT2 cells into post-mitotic neurons, and to our knowledge, this is the first report of a human neuronal cell line that expresses high levels of CYP46A1 mRNA.

Our previous work, pointed to Sp transcription factors as major regulators of *CYP46A1* basal expression (Milagre *et al.* 2008). Combination of Sp1, Sp3 and Sp4 overexpression studies in *Drosophila* SL2 cells, and systematic promoter mutagenesis identified Sp3 and Sp4 binding to four GC-boxes as required and sufficient for high levels of promoter activity. Moreover, EMSA demonstrated that the transcription factors Sp1, Sp3 and Sp4 bind to the GC-boxes on the *CYP46A1* proximal promoter, and that Sp3 and Sp4 were the major components of the protein-DNA complexes observed in primary rat cortical extracts. Thus, it was our hypothesis that Sp1, Sp3, and Sp4 functionally interact with the *CYP46A1* endogenous promoter, and that a shift that would increase the (Sp3+Sp4)/ Sp1 ratio would result in the transcriptional activation this gene. Indeed, it is well documented that changes in the relative levels of Sp1/Sp3/Sp4 are one of the mechanisms by which the Sp transcription factors regulate gene expression (Lee *et al.* 2004, Magee *et al.* 2005, Mao *et al.* 2006). Analysis of the expression levels of Sp proteins during the differentiation process revealed that there is a 50 % decrease in Sp1 protein levels in NT2N post-mitotic neurons, while the levels of Sp3 and Sp4 remained unchanged. Interestingly, we did not detect any significant decrease in Sp1 mRNA levels (data not shown). Moreover, we only detected the decrease in Sp1 protein after addition of mitotic inhibitors (cytosine D-arabinofuranoside, fluorodeoxyuridine and uridine) to the cell culture. We have also detected a decrease of Sp1 protein levels when we treated SH-SY5Y neuroblastoma cells with the DNMT inhibitor, 5'-Aza-2'-deoxycytidine (Milagre *et al.* 2010) (results discussed in Chapter 4), a drug that has also been shown to induce cell cycle arrest (Lavelle *et al.* 2003). Although Sp1 has been described to play an important role in mitosis, by activating the transcription of the genes encoding D-type cyclins, cyclin E, Cdk2, E2F-1 and c-Myc, which are key factors for progression through G1-phase and entry into S-phase (Wierstra 2008), it has not been thoroughly investigated

how mitotic inhibitors may affect Sp1 protein levels. Nevertheless, several evidences support the role of posttranslational modifications in the regulation of Sp1 stability during mitosis (Chuang *et al.* 2008, Spengler *et al.* 2008). Indeed, a shift between activator and repressor function is a common feature of transcription factors, and an aspect of this feature includes the coupling of ubiquitin / proteasome mediated degradation.

Sp1 was one of the first transcription factors purified and cloned from mammalian cells (Dynan and Tjian, 1983), and it is generally considered as a factor that determines the core activity of promoter by a direct interaction with factors at the basal transcription machinery, including cooperation with several transcriptional activators, such as CRSP, p300/CBP, etc (Owen *et al.* 1998, Ryu *et al.* 1999). Corresponding to its function in the activation of many house-keeping genes, Sp1 is widely expressed in human tissues. An increasing amount of data, however, accumulates that Sp1-dependent transcription might also be involved in the regulation tissue-specific expression, namely of brain-specific genes (Chen *et al.* 2007, Xu & Carr 2001, Liu *et al.* 2003). Nevertheless, Mao and co-workers have found that Sp1 protein is nearly absent in forebrain neurons, and have raised the question whether substitution of the ubiquitous Sp1 by the brain enriched Sp4 in neuronal cells, underlies the cell-type specificity expression in the CNS (Mao *et al.* 2007, Ishimaru *et al.* 2007).

In order to understand if the shift observed in the ratio of the different Sp proteins in post-mitotic neurons was correlated with qualitative changes in the binding of these transcription factors to the *CYP46A1* proximal promoter in the context of native chromatin, we performed ChIP using in NT2N cells, that express high levels CYP46A1 mRNA, and compared the results with those obtained in undifferentiated NT2 d0 cells. ChIP assays provided strong evidence in support of a correlation between the increase in CYP46A1 mRNA levels, the decrease in Sp1 protein levels, and a 50% decrease in the Sp1 recruited to the proximal promoter of this gene.

These results raised the question of why displacing Sp1 from the *CYP46A1* promoter can up-regulate this gene. Although, our ChIP results strongly point to the dissociation of Sp1 from the proximal promoter, there was no overall increase of Sp3 and Sp4 binding to the promoter. This implies that either in undifferentiated NT2 cell, Sp1 is involved in the recruitment of HDAC or co-repressors complexes, or that the release of Sp1 in NT2N may induce a more favorable conformation of Sp3 and Sp4 to bind to associated co-activators or brain-specific transcription factors. Another possibility is that a Sp1 free promoter favors the recognition of *cis*-acting sites by other transcription factors.

Indeed, Sp1 has been described to bind directly to HDAC1 and other co-repressors (Doetzlhofer *et al.* 1999, Zhang & Dufau 2002, Lagger *et al.* 2003, Bu & Gelman 2007), and to be implicated in the HDAC-dependent repression of the p21 (Lagger *et al.* 2003), Src-suppressed C kinase substrate (Bu & Gelman 2007), thymidine kinase (Doetzlhofer *et al.* 1999) and human telomerase reverse transcriptase (Hou *et al.* 2002) promoters. Surprisingly, we did not detect HDAC1 and 2 recruited to the *CYP46A1* promoter in undifferentiated NT2 cells, nor did we detect a significant increase in promoter histone acetylation in NT2N cells (data not shown).

On the other hand, our previous results showed that the highest activation of the *CYP46A1* promoter was achieved by co-transfection of Sp3 and Sp4 (Milagre *et al.* 2008), suggesting that Sp1 might be a less potent transcriptional activator of this gene. Nevertheless, direct interactions between Sp3 and Sp4 and other nuclear proteins cannot be excluded as a mechanism of *CYP46A1* activation. Indeed, although there is little information about putative multimerization regions of Sp4 protein available for protein-protein interactions in the DNA-bound state, this transcription factor has been shown to activate expression of photoreceptors-specific genes, by synergizing with Crx, and it has also been shown that Sp3 and possible Sp1 may inhibit this Sp4-mediated transcriptional activation (Lerner *et al.* 2005).

It is commonly accepted that Sp1, Sp3 and Sp4 can have both positive and negative effects on DNA binding and transcriptional activity, and that this dichotomy is likely to reflect, at least in part its posttranslational modifications or the promoter context. Indeed, Sp4 has been described to have opposite effects on the regulation of neurotrophin-3 (NT-3) and the high affinity NT-3 receptor TrkC promoters: overexpression of Sp4 reduced NT3 promoter activity and increased TrKC promoter activity whereas knockdown of Sp4 increased expression of NT3 and reduced expression of TrKC (Ramos *et al.* 2009). Interestingly, it has been shown that in the human plasma membrane-associated sialidase *locus*, when Sp1 and Sp3 bind to a particular response element, they can promote transcription from transcription start site 1, while repressing the brain-specific transcription start site 0 (Yamaguchi *et al.* 2010).

An alternative hypothesis is that an additional transcriptional regulator might be tethered to overlapping sites and/or Sp1 sites, once Sp1 levels are decreased. In fact, such a mechanism has been suggested in the case of the p14ARF promoter, where *in vivo* displacement of Sp1 at the overlapping sites by E2F1 requires a threshold E2F1 to overcome Sp1 occupation (Zhang *et al.* 2009). Probably this is not the case for *CYP46A1*,

since when we have used nuclear extracts from NT2N cells, the supershift analysis did not detected any proteins other than Sp proteins bound the GC-boxes, that we had previously identified has essential for basal promoter activity (data not shown).

Since it would be interesting to analyse if an increase in the (Sp3+Sp4)/ Sp1 ratio was a common feature in the context of native chromatin in neuronal promoters other than *CYP46A1,* we have selected four genes – MOR, reelin, GRIN1 and GRIN2A, that have been shown to be expressed in the human brain (Bai & Kusiak 1995, Chen *et al.* 2002, Arvidsson *et al.* 1995) and to be regulated by Sp transcription factors (Traynelis *et al.* 2010, Wei & Loh 2010, Levenson *et al.* 2008). Interestingly, at the reelin promoter we have detected a complete switch in binding between Sp1 and Sp4; indeed, we detected the association of Sp1 and Sp3, but not Sp4 in the undifferentiated cells, while only Sp3 and Sp4 are bound to the promoter in the NT2N post-mitotic neurons. Chen and co-workers had previously shown that induction of the reelin promoter with RA in NT2 cells was accompanied by higher amounts of Sp1 and Pax6 binding to the proximal promoter (Chen *et al.* 2007). Nevertheless, these authors have used NT2 cells treated just for six days with RA, and therefore, these results cannot be directly compared with the results we obtained with post-mitotic NT2N neurons.

Analysis of Sp binding to the other selected gene promoters, MOR, GRIN1 and GRIN2A, revealed that, like in other cell types, binding of Sp proteins to DNA in a neuronal chromatin environment appears highly dependent on promoter context. Indeed, we could not identify any conserved alteration in the Sp binding patterns to the formerly mentioned promoter regions, nor did we detect the association of Sp4 in differentiated NT2N cells.

Herein, we demonstrated a significant increase in CYP46A1 mRNA levels after neuronal differentiation of NT2 cells, which is accompanied by a decrease in the Sp1 associated to the proximal promoter of this gene. Our data also point to the fact that, contrary to what we initially hypothesize, the dissociation of Sp1 from the promoter regions of neuronal Sp-regulated genes, is not a common feature, suggesting that although the Sp1 levels are significantly decreased after neuronal differentiation, Sp1-dependent transactivation of specific neuronal promoter is most likely to occur, and that there is still much to learn about the Sp protein regulatory functions in neurons

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NEURONAL DIFFERENTIATION IS ASSOCIATED WITH A CHANGED BALANCE BETWEEN CHOLESTEROL SYNTHESIS AND DEGRADATION BY THE CYP46A1 AND CYP27A1 MEDIATED PATHWAYS

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Manuscript in preparation

4.1 Abstract

Brain cholesterol metabolism in the CNS is distinct from that in other tissues, since exchanges of cholesterol through the BBB do not occur. Cholesterol homeostasis is maintained by a series of interdependent processes involving synthesis, transport and catabolism, which are controlled by several proteins also expressed in other tissues. Nevertheless, brain cholesterol elimination depends on a neuronal-specific cytochrome P450, CYP46A1, and the lack of a cellular model that expresses high levels of this enzyme has limited the insight into how human post-mitotic neurons handle cholesterol. Herein, we aimed to assess if differentiated Ntera2/clone D1 (NT2) cells could be a useful model to study the cellular mechanisms involved in neuronal cholesterol homeostasis. We have shown that NT2N post-mitotic neurons express key genes involved in brain cholesterol synthesis, synthesis regulation and uptake, namely HMG-CoA synthase, HMG-CoA reductase, SREBP2 and LDLr. Moreover, the expression profiles observed during neuronal differentiation are in accordance with what is thought to occur *in vivo*, namely the decrease in the levels of cholesterol synthesizing enzymes. Furthermore, this is the first time that CYP46A1 protein and 24OHC have been detected in human neuronal cells in culture. CYP46A1 protein is upregulated in post-mitotic neurons, with a concomitant decrease in CYP27A1 protein levels. We observed an increase in the ratio between 24OHC and 27OHC levels throughout neuronal differentiation, which suggests that progenitor cells eliminate cholesterol in the form of 27OHC, while neurogenesis induces a change to the 24-hydroxylase-dependent elimination pathway. Moreover, CYP39A1 and CYP7B1, the enzymes responsible for metabolizing 24OHC and 27OHC, are also up-regulated in post-mitotic neurons, which can partly explain the lack of enzyme-product correlation through the differentiation process. Taken together, our results demonstrate that differentiated NT2 cells are a valuable cell model for the study of cholesterol homeostasis in human neurons.

4.2 Introduction

Cholesterol plays an essential role in central nervous system (CNS) structure and function, being required for proper myelination, stabilization and organization of axonal microtubules, dendritic differentiation and synaptic plasticity (Saher *et al.* 2005, Fan *et al.* 2001, Fan *et al.* 2002, Mauch *et al.* 2001). Since the blood-brain barrier (BBB) prevents uptake of cholesterol from the circulation, brain cholesterol is essentially synthesized *in situ* (Bjorkhem & Meaney 2004). Cholesterol synthesis in the developing CNS is relatively high, but declines to very low levels in the adult (Dietschy & Turley 2004). This is explained by a highly efficient recycling of brain cholesterol, which has an unusual long half-life that has been estimated to be at least 5 years (Bjorkhem *et al.* 1998). Frank Pfrieger has put forward the hypothesis that neurons switch from production of cholesterol during the embryonic stage to rely constitutively on cholesterol delivery by astrocytes via lipoproteins after birth (Pfrieger 2003). Indeed, evidence for a cholesterol shuttle from astrocytes to neurons has been presented (Mauch *et al.* 2001), and results obtained from *in vitro* systems demonstrated that in rodents, neurons and glial cells present distinct profiles of biosynthetic enzymes, post-squalene precursors and cholesterol metabolites (Nieweg *et al.* 2009). Nevertheless, it is thought that due to the structural and functional specialization of brain regions, some neurons may depend entirely on an external cholesterol supply while others may be autonomous (Pfrieger 2003). Indeed, HMG-CoA reductase and 7- dehydrocholesterol reductase, are expressed in neurons throughout the adult mouse brain (Korade *et al.* 2007). *In situ* data provided by the Allen Brain Mouse Atlas, has revealed that the expression of cholesterol homeostasis genes are preferentially targeted to neuronal hippocampal pyramidal and granule layers (Valdez *et al.* 2010).

Despite the efficiency of the cholesterol recycling machinery, the rate of cholesterol synthesis in the adult brain is larger than the rate of accumulation, therefore a mechanism by which excess cholesterol is excreted has to exist. The major mechanism of brain cholesterol elimination involves the conversion of cholesterol into 24(*S*)-hydroxycholesterol (24OHC), which readily crosses the BBB (Lütjohann *et al.* 1996, Bjorkhem *et al.* 1997, Bjorkhem *et al.* 1998). The enzyme responsible for 24(*S*)-hydroxylation of cholesterol is a cytochrome P450 - CYP46A1 - that is almost exclusively expressed in neurons (Lund *et al.* 1999, Bretillon *et al.* 2007). Interestingly, inactivation of *Cyp46a1* resulted in a decrease of 24OHC levels in the brain (~60%), that

was associated with a selective reduction of cholesterol synthesis (~40%), suggesting a close relation between synthesis and metabolism of cholesterol in the CNS (Lund *et al.* 2003). Another oxysterol that may be involved in brain cholesterol homeostasis is 27-hydroxycholesterol (27OHC). There is a flux of 27OHC from the circulation into the brain of the same magnitude as the flux of 24OHC in the opposite route (Heverin *et al.* 2005). Both oxysterols are potent suppressors of cholesterol synthesis and are ligands for LXRα and β, nuclear receptors which play an important role in lipid homeostasis in the CNS (Wang *et al.* 2002).

Although several human derived neuroblastoma cell lines express cholesterol synthesizing enzymes, the lack of a cellular model that expresses high levels of CYP46A1 enzyme has limited the insight of how human post-mitotic neurons handle cholesterol. Moreover, comparison of the human and rat *CYP46A1* promoter sequences revealed significant differences, upstream of the core promoter (Milagre *et al.* 2008), suggesting that results from studies in primary cortical and hippocampal neurons isolated from mouse embryos may not reflect what happens in human brain.

We have demonstrated that differentiation of Ntera2/clone D1 (NT2) cells by retinoic acid into post-mitotic neurons (NT2N), might be a good *in vitro* system to study cellular mechanisms involved in human neuronal cholesterol metabolism, since after differentiation we could observe a significant increase in CYP46A1 mRNA levels (results presented in Chapter 3). Herein we have examined cholesterol metabolites, the expression of sterol hydroxylases and of other genes important for cholesterol synthesis, catabolism and transport during the differentiation of NT2 cells into post-mitotic neurons. Our results demonstrate that the key players in maintaining cholesterol homeostasis in the brain are expressed in NT2N, and that there is a shift in the 24OHC / 27OHC ratio throughout neuronal differentiation.

4.3 Methods

4.3.1 Cell Culture

NTERA-2cl.D1 testicular embryonal carcinoma cells (NT2) were cultured as previously described (Chapter 3, section 3.3.1).

4.3.2 Expression Analysis

Total cell RNA was extracted using Trizol Reagent (Invitrogen Carlsbad, CA, USA) following manufacturer's instructions. Real-Time PCR analysis for CYP46A1 mRNA levels was performed as described previously (Nunes *et al.* 2010). Real-Time PCR analysis for HMG-CoA Reductase, HMG-CoA Synthase, SREBP-2, LDLr, CYP7B1 and CYP39A1 was performed using SYBR green Master Mix in an ABI 7300 sequence detection system (Applied Biosystems) and specific primers (Table 4.1). Results presented were obtained from at least three individual experiments and each sample was assayed in triplicate. CYP46A1 and CYP27A1 mRNA levels were normalized to the level of β-actin and expressed as pg of CYP mRNA per ng of β-actin mRNA. HMG-CoA Reductase, HMG-CoA Synthase, SREBP-2 LDLr, CYP7B1 and CYP39A1 were normalized to the level of β-actin as fold change from controls, using the $^{\Delta\Delta}$Ct method.

Table 4.1 – List of primers used for qPCR.

Gene	Primer / TaqMan Assay	Sequence (5'→ 3') / Source
CYP46A1	CYP46A1 HS00198510_M1	Applied Biosystems Ref:4331182
CYP27A1	CYP27A1 HS01026016_M1	Applied Biosystems Ref:4331182
β Actin	ACTB HS99999_M1	Applied Biosystems Ref:4310881E-060521
HMGCoA	HuHMGCoARedf	5'-ATAGGAGGCTACAACGCCCAT-3'
Reductase	HuHMGCoARedr	5'-TTCTGTGCTGCATCCTGTCC-3'
HMGCoA	HuHMGCoASynf	5'-GGCACAGCTGCTGTCTTCAAT-3'
Synthase	HuHMGCoASynr	5'-ACCAGGGCATACCGTCCA T-3'
SREBP2	HuSREBP2f	5'-CAGCTGCACATCACAGGGAA-3'
	HuSREBP2r	5'-GTACATCGGAACAGGCGGAT-3'
LDLr	HuLDL-Rf	5'-CAGATATCATCAACGAAGC-3'
	HuLDL-Rr	5'-CCTCTCACACCAGTTCACTCC-3'
CYP7B1	qPCRHuCYP7B1f	5'-CAGCCTAATCTGCCTAGAAAGCAGC-3'
	qPCRHuCYP7B1r	5'-CGCACACAGTAGTCCCCGGTC-3'
CYP39A1	qPCRHuCYP39A1f	5'-CCAGTGTCCTGCAAGGTGGTTTGC-3'
	qPCRHuCYP39A1r	5'-GCTGGGGGACACCCACCAAAT-3'
β Actin	qPCRHuACTINf	5'-CTGGAACGGTGAAGGTGACA-3'
	qPCRHuACTINr	5'-AAGGGACTTCCTGTAACAATCCA-3'

4.3.3 Western blot analysis

Mitochondrial and microsomal extracts were prepared as described previously with minor modifications (Mast *et al.* 2010). In brief, confluent cells were washed with cold PBS containing 1 mM Pefabloc and centrifuged at 200 *g* for 10 minutes. The supernatants were discarded, the pellets resuspended in 6 volumes (v/v) of homogenization buffer (50

mM Tris-Cl (pH7.4), 250 mM sucrose, 5 mM MgCl2, 1 mM Pefabloc, 1 mM DTT and protease inhibitor cocktail), incubated on ice for 2 minutes and homogenized in a glass homogenizer. The crude homogenate was centrifuged at 1000 *g* for 15 minutes. The supernatant was collected and kept in a separate tube on ice. The pellet was resuspended and centrifuged again at 1000 *g* for 15 minutes. The supernatants were combined and centrifuged at 10000 *g* for 20 minutes. The supernatant was collected and kept in a separate tube on ice and the pellet was further resuspended in 3 volumes (V/V) of homogenization buffer and centrifuged. The resulting pellet containing the mitochondrial fraction was resuspended in an appropriate volume of homogenization buffer, whereas the combined supernatants were further centrifuged at 100000 *g* for 60 minutes. The resulting pellets containing the microsomal fraction were resuspended in an appropriate volume of homogenization buffer. All steps were carried out at 4ºC. Mitochondrial and microsomal extracts were subject to 10% or 12.5% SDS-PAGE gels and electroblotted onto Immobilon P (Millipore, Bedford, MA, USA). After visualization of the transferred proteins by amido black staining, the membranes were incubated with specific antibodies. Results were quantified using the Quantity One version densitometry analysis software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

4.3.4 Sterol extraction and gas chromatography - mass spectrometry (GC-MS) analysis

Total sterols from cell pellets and culture medium were extracted and analyzed by isotope dilution–mass spectrometry using deuterium-labeled oxysterols as internal standards, as previously described (Dzeletovic *et al.* 1995).

4.3.5 Statistical Analysis

Statistical analysis was performed using the Kruskal-Wallis test with Dunn's post-hoc test or the ANOVA one-way test with the Tukey HSD post-hoc test or the Tukey HSD for unequal N (Spjotvoll/Stoline test). All analysis was performed using the STATISTICA (data analysis software system), version 9.1 StatSoft, Inc. (2010) or Prism 5.0 software (GraphPad Software).

4.4 Results

4.4.1 Expression pattern of genes involved in cholesterol synthesis, transport and catabolism during NT2 neuronal differentiation

In order to evaluate if the NT2 cell culture system could be a useful system to study the cellular mechanisms involved in human neuronal cholesterol metabolism, we have examined by real-time PCR the mRNA levels of genes involved in cholesterol synthesis, transport and elimination, during NT2 neuronal differentiation (Figure 4.1). Our results show a significant decrease of both HMG-CoA synthase and HMG-CoA reductase mRNA levels during NT2 differentiation (ANOVA one-way test: $F = 48.78$, $df = 3$, $p < 0.001$ and ANOVA one-way test: $F = 13.8$, $df = 3$, $p < 0.01$, respectively). Post-hoc comparisons revealed significant differences between NT2 d0 and all the analysed time-points for HMG-CoA synthase (Tukey HSD, $p < 0.001$) and for HMG-CoA reductase (Tukey HSD, $p < 0.05$ for NT2 d21; $p < 0.01$ for NT2N R7d and NT2NR15d). These data suggest that human NT2 cells suppress cholesterol biosynthesis during neuronal differentiation; nevertheless we did not observe any significant difference on the mRNA levels of SREBP2, a transcription factor that regulates the expression of several genes closely related to cholesterol metabolism. LDLr mRNA levels significantly decrease during RA administration (ANOVA one-way test: $F = 32.83$, $df = 3$, $p < 0.001$), whereas in post-mitotic neurons the mRNA levels of this receptor tend to increase, reaching levels similar to those of NT2d0.

The analysis of sterol 27-hydroxylase (CYP27A1) and cholesterol 24-hydroxylase (CYP46A1), revealed that CYP27A1 mRNA levels were significantly induced by RA treatment, reaching a peak at d21 (ANOVA one-way test: $F = 43.42$, $df = 3$, $p < 0.001$; Tukey HSD, $p < 0.001$), and then gradually decrease. Indeed, post-hoc comparisons revealed significant differences between NT2 d21 and both NT2N R7 and R15d (Tukey HSD, $p < 0.001$), although the mRNA levels do not reach those observed at NT2 d0. As expected, mRNA levels for the neuronal specific CYP46A1 significantly increase throughout differentiation (ANOVA one-way test: $F = 20.04$, $df = 3$, $p < 0.001$).

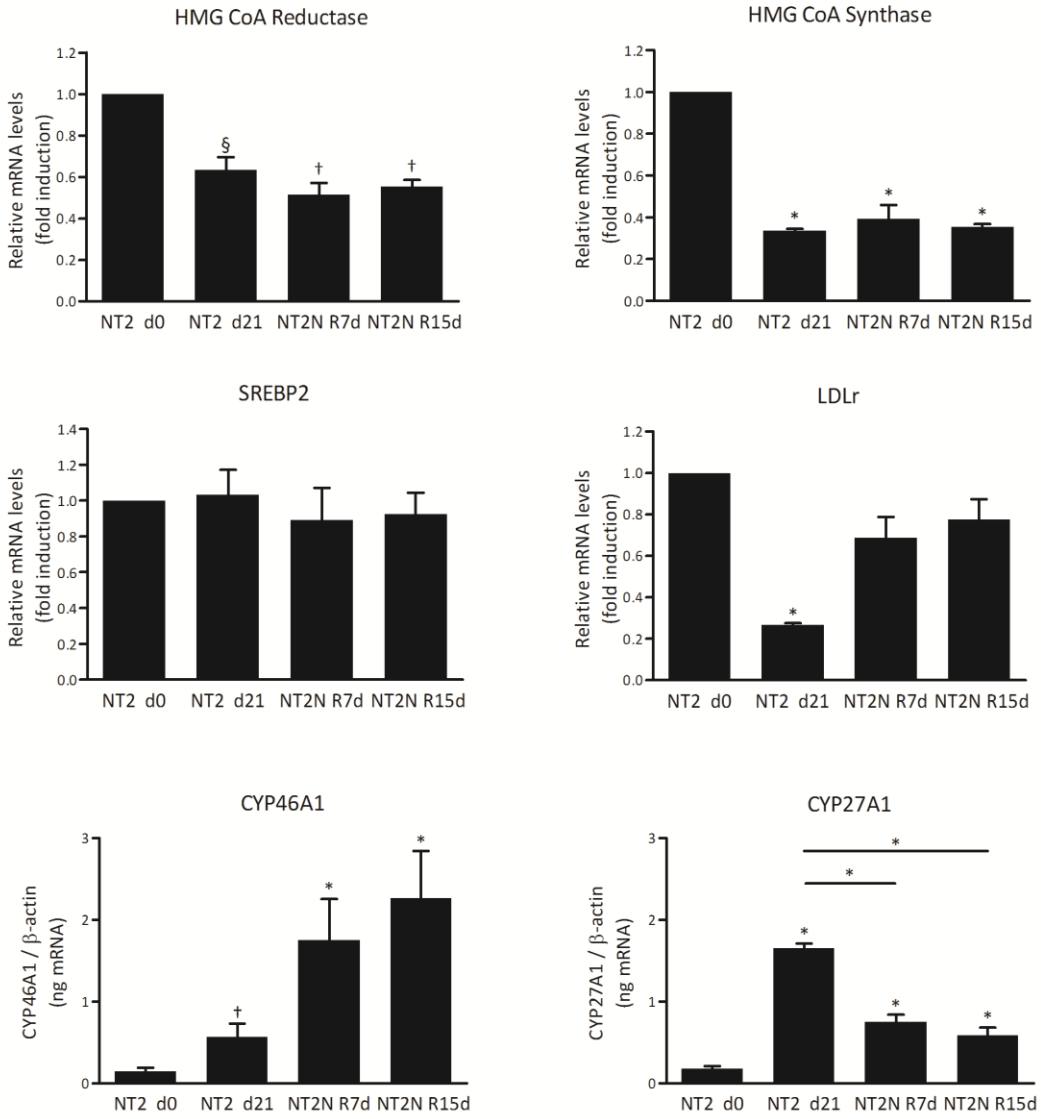

Figure 4.1. mRNA expression analysis of sterol hydroxylases and other key genes in cholesterol homeostasis during differentiation of NT2 cells. Real-time PCR analysis of steady-state mRNA transcript levels in NT2 cells during differentiation. Values were normalized to the internal standard β-actin. Data represent means ± SEM from at least three individual experiments. CYP46A1 and CYP27A1 mRNA levels were normalized to the level of β-actin and expressed as pg of CYP mRNA per ng of β-actin mRNA. HMG-CoA Reductase, HMG-CoA Synthase, SREBP-2 and LDLr were normalized to the level of β-actin as fold change from controls (§$p < 0.05$; †$p < 0.01$; *$p < 0.001$).

4.4.2 CYP46A1 and CYP27A1 protein levels during NT2 neuronal differentiation

To confirm that the increase in CYP27A1 and CYP46A1 mRNA levels are reflected at the protein level, we performed western blot analysis of mitochondrial and microsomal extracts (respectively) obtained from cells collected at different time points of the

differentiation (Figure 4.2). We observed that, CYP46A1 protein can only be detected at the final stages of NT2 differentiation, and that the increase in CYP46A1 protein levels occurs concomitantly with the increase in mRNA levels. CYP27A1 protein is readily detected at NT2 d0 and the levels of this enzyme slightly decrease at NT2N R15d (Kruskal-Wallis test, $p < 0.05$).

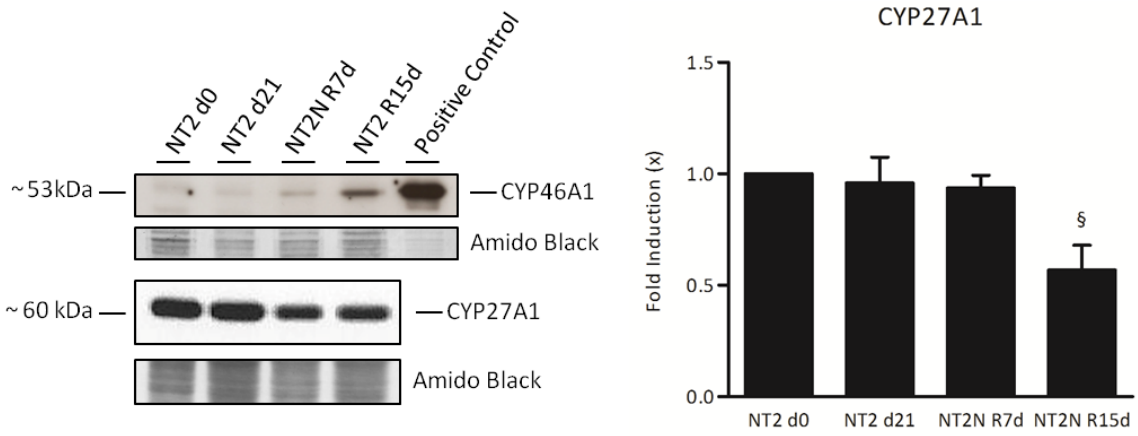

Figure 4.2. CYP46A1 and CYP27A1 protein levels during differentiation of NT2 cells. A) Western blot analysis of CYP46A1 and CYP27A1 protein levels in NT2 cells during neuronal differentiation. Mouse brain microsomes were used as a positive control, for the CYP46A1 western blot. Microsomal and mitochondrial extracts were subjected to SDS-PAGE, transferred to PVDF membranes, and incubated with specific antibodies. Protein loading control using Amido Black staining of the membrane is shown in the bottom panel. B) Quantification of CYP27A1 protein levels. Data represent means ± SEM and are expressed as fold change relative to control cells (§$p < 0.05$). The results shown are representative of those obtained in three independent cultures.

4.4.3 Quantification of cholesterol metabolites during NT2 neuronal differentiation

We measured the levels of cholesterol metabolites extracted from the medium and cellular fraction throughout the differentiation process of the NT2 cells by GC-MS analysis. Oxysterols can be formed by the action of specific enzymes or by auto-oxidation of the cholesterol molecule. 24OHC and 27OHC have been demonstrated to be enzymatically formed by specific sterol hydroxylases, CYP46A1 and CYP27A1, respectively, whereas there is little or no evidence that these can be products of cholesterol auto-oxidation (Bjorkhem & Diczfalusy 2002, Schroepfer 2000). Our analysis revealed measurable levels of both 24OHC (0.13 ± 0.02 ng of 24OHC/µg of total cell cholesterol in cell pellets and 0.29 ± 0.2 ng of 24OHC/µg of total cell cholesterol from

the medium) and 27OHC (0.65 ± 0.07 ng of 27OHC/µg of total cell cholesterol extracted from cell pellets and 2.3 ± 1.0 pg of 27OHC/µg of total cell cholesterol, from the medium) at d0 of differentiation (Figure 4.3). We observed that, although there is a significant increase in mRNA and protein levels of CYP46A1, the levels of 24OHC extracted from cell pellets and secreted to the medium were not altered (Figure 4.3). The levels of 27OHC extracted from cell pellets (Figure 4.3A) significantly decreased in the replate stages to levels lower than the control (0.29 ± 0.09 ng and 0.12 ± 0.03 ng of 27OHC/µg of total cell cholesterol in NT2N R7d and NT2N R15d, respectively; ANOVA one-way test: F = 51.89, df = 3, $p < 0.001$). The levels of 27OHC secreted to the medium (Figure 4.3B) were not altered at NT2 d21 (2.4 ± 0.87 pg of 27OHC/µg of total cell cholesterol), but also decreased in the replate stages to levels significantly lower than those observed at the NT2 d0 (0.46 ± 0.12 and 0.2 ± 0.05 pg of 27OHC/µg of total cell cholesterol in NT2N R7d and R15d, respectively) (ANOVA one-way test: F = 109.67, df = 3, $p < 0.001$).

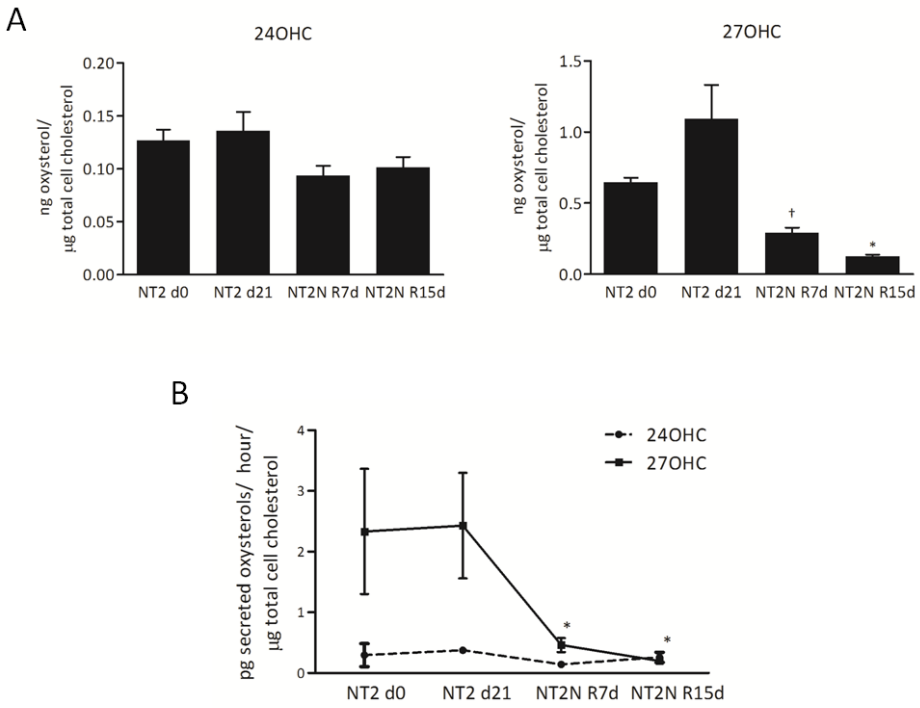

Figure 4.3. Oxysterol levels during NT2 neuronal differentiation. Levels of oxysterols extracted from cell pellets (A) and medium (B) were determined at day 0 (NT2 d0) and day 21 (NT2 d21) of RA treatment, and at day 7 (NT2N R7d) and day 15 (NT2N R15d) after cells were replated in PDL-MG coated plates, as described in materials and methods. Data are expressed as ng oxysterol/ µg total cholesterol (A) and pg secreted oxysterol/ hour/ total cholesterol (B) and represent mean values ± SD of at least three independent experiments (§$p < 0.05$; †$p < 0.01$; *$p < 0.001$).

4.4.4 Shift in the 24OHC / 27OHC levels during NT2 neuronal differentiation

Reflecting what happens in the adult brain in physiological conditions, where only a small production of 27OHC occurs in CNS (Bjorkhem *et al.* 2010), our results showed an increase in the ratio between 24OHC and 27OHC during neuronal differentiation (Figure 4.4). Indeed, in the cellular fraction the 24OHC/ 27OHC ratio increased from approximately 0.2 at d0 to 1 at NT2N R15d (ANOVA one-way test: F = 20.24, df = 3, $p < 0.001$), and the ratio between both oxysterols secreted to medium increased from approximately 0.12 to 1.39 (ANOVA one-way test: F = 21.82, df = 3, $p < 0.001$).

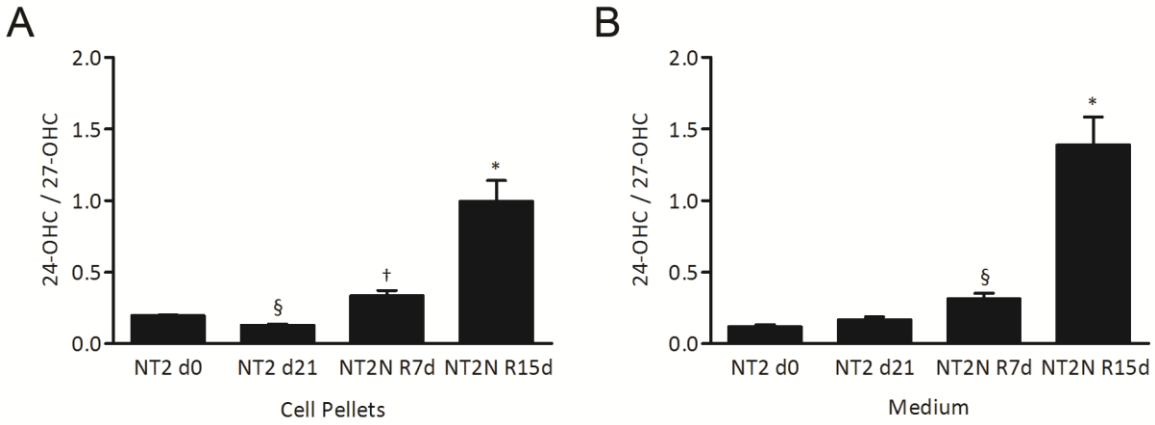

Figure 4.4. Ratio between 24- and 27- hydroxycholesterol during NT2 neuronal differentiation. The ratios of 24OHC and 27OHC extracted from cell pellets (A) and medium (B) were calculated at day 0 (NT2 d0) and day 21 (NT2 d21) of RA treatment, and at day 7 (NT2N R7d) and day 15 (NT2N R15d) after cells were replated in PDL-MG coated plates, as described in materials and methods. Data represent mean values ± SD of at least three independent experiments (§$p < 0.05$; †$p < 0.01$; *$p < 0.001$).

4.4.5 Expression pattern of genes involved in oxysterol metabolism during NT2 neuronal differentiation

We have demonstrated a lack of enzyme-product correlations when we compared the changes in 27OHC levels with the changes in CYP27A1 protein levels. Indeed, although we did observe a decrease in CYP27A1 in NT2N R15d, this decrease was far from being as drastic as the decrease observed in 27OHC levels. Moreover, the increase in CYP46A1 mRNA/ protein levels was also not correlated with an increase in 24OHC levels. Since CYP7B1 and CYP39A1 are known to metabolize 27OHC and 24OHC, respectively, we have investigated the expression levels of these enzymes. We observed that both CYP7B1 as well as CYP39A1 mRNA levels significantly increased at NT2 d21 (Figure 4.5)

(ANOVA one-way test: F = 16.16, df = 3, $p < 0.001$ and ANOVA one-way test: F = 49.40, df = 3, $p < 0.001$). Afterwards, mRNA levels start to decrease to levels that are still significantly higher than control levels.

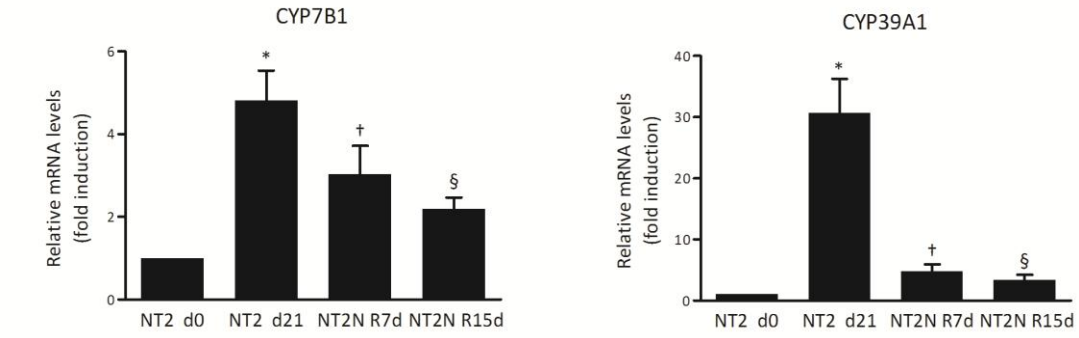

Figure 4.5. mRNA expression analysis of CYP7B1 and CYP39A1 during differentiation of NT2 cells. Real-time PCR analysis of steady-state mRNA transcript levels in NT2 cells during differentiation. Data represent means ± SEM from at least three individual experiments. Values were normalized to the internal standard β-actin and are represented as fold change from controls (§$p < 0.05$; †$p < 0.01$; *$p < 0.001$).

4.5 Discussion

A large effort has been made in the last decade to unravel the mechanisms underlying cholesterol metabolism in the brain. There are significant quantitative and qualitative differences in cholesterol homeostasis in rodents, when compared to humans (Dietschy & Turley 2004, Dietschy & Turley 2001, Heverin *et al.* 2005). Thus, a better understanding of cholesterol homeostasis and the consequences of its deregulation in neurodegenerative disorders would be greatly enhanced by human cellular models, where the key players involved in the control of brain cholesterol synthesis, transport and catabolism are expressed.

Our work has focused on the characterization of NT2 post-mitotic neurons as a model for the study of human CNS cholesterol homeostasis. In this work, we have assessed the expression profiles of several genes coding for key enzymes involved in cholesterol synthesis (HMG-CoA synthase and HMG-CoA reductase) and synthesis regulation (SREBP2), uptake (LDLr) and catabolism (CYP27A1 and CYP46A1), during neuronal differentiation. The rate limiting enzymes in cholesterol biosynthesis - HMG-CoA synthase and HMG-CoA reductase - are downregulated during NT2 differentiation,

which is in agreement with the decreased cholesterol synthesis known to occur in the adult brain (Dietschy & Turley 2004). Moreover, we detected high mRNA levels of SREBP2, a key transcriptional regulator of genes involved in cholesterol metabolism, namely HMG-CoA synthase, HMG-CoA reductase and LDLr. Indeed, in depleted cholesterol conditions, SREBP2 suffers proteolytic processing, the active peptides are translocated to the nucleus and enhance gene transcription (Brown & Goldstein 1997). It is however well established that, although cholesterol levels control SREBP activity, fluctuations in cellular cholesterol do not alter SREBP transcription (Goldstein & Brown 1990). Therefore, the fact that we did not detect any significant change in SREBP2 mRNA levels throughout differentiation of NT2 cells was not unexpected. No significant differences were detected between LDLr mRNA levels in NT2 d0 and NT2 R15d, which is in agreement with what is described *in vivo*; in fact, Hanaka and co-workers have shown that LDLr mRNA levels in the adult brain are similar to those observed in the embryonic brain (Hanaka *et al.* 2000).

Sterol hydroxylases also play an important role in the maintenance of cholesterol homeostasis, since they can convert cholesterol into oxysterols which are side chain oxidized sterols that have the ability to pass cell membranes at a much faster rate than cholesterol itself (Bjorkhem & Diczfalusy 2002). Moreover, oxysterols can act as important ligands to various receptors involved in lipid metabolism (Schroepfer 2000). CYP27A1 is a ubiquitously expressed cytochrome P450 that is able to convert cholesterol into 27OHC (Babiker *et al.* 1997, Russell 2000). It has been shown that mutations in the *CYP27A1* gene, that prevent the formation of 27OHC, cause cerebrotendinous xanthomatosis a disease that is characterized by the accumulation of cholesterol metabolites in the brain and peripheral tissues (Russell 2000), which has a deleterious effect on cognition (Kim *et al.* 2009b). On the other hand, CYP46A1 is a brain-specific cytochrome P450 that was found to be responsible for the conversion of cholesterol to 24OHC (Lund *et al.* 1999). This is thought to be the major mechanism by which excess cholesterol is excreted from the brain (Bjorkhem *et al.* 1997). In fact, mice with a disruption in this gene exhibit severe deficiencies in spatial, associative, and motor learning and have impaired hippocampal long-term potentiation (Kotti *et al.* 2006). Here, we have confirmed our previously results, and show that if NT2N cells are left in culture with mitotic inhibitors for two weeks, CYP46A1 mRNA reaches slightly higher levels than those previously observed at NT2N R7d, which is reflected in a pronounced increase in protein levels. CYP27A1 mRNA levels significantly increase at NT2 d21, and then

start to decrease to levels slightly above the control. Indeed, CYP27A1 has been previously described as a RAR/ RXR-target gene in myeloid cells (Szanto *et al.* 2004, Quinn *et al.* 2005). Nevertheless, we did not observe a significant increase in CYP27A1 protein levels at NT2 d21, but instead we observed a significant decrease at NT2N R15d, when compared with undifferentiated cells. These results suggest that undifferentiated cells may dependent upon the CYP27A1 elimination pathway whereas, post-mitotic neurons depend on cholesterol efflux in the form of 24OHC.

Since there was such a marked modulation in CYP27A1 and CYP46A1 levels throughout the differentiation process, we also investigated the levels of their products - 27OHC and 24OHC. Surprisingly, we observed that there is no change in the levels of 24OHC, both in the cell pellets and in the medium. In contrast, the levels of 27OHC secreted to the medium and in cells pellets decreased in NT2N R15d to levels lower than those observed at the NT2 d0.

Although the levels of CYP46A1 do not correlate with the 24OHC detected in either cell pellets or the medium, there is a significant shift in the ratio between 24OHC and 27OHC that favours 24OHC in the final stages of differentiation of the NT2 cells into neurons. This may reflect what happens in the adult brain in non-pathological conditions where only a small production of 27OHC occurs in CNS (Bjorkhem *et al.* 2010), while the majority of 24OHC in the organism is produced in the brain (Lütjohann *et al.* 1996). One may speculate that neurogenesis leads to a higher utilization of the CYP46A1 pathway to eliminate cholesterol in the form of 24OHC, whereas the multipotent progenitor cells eliminate cholesterol in the form of 27OHC.

The lack of enzyme-product correlation observed when we compared the changes in 24OHC and 27OHC levels with the changes in CYP46A1 and CYP27A1 protein levels, led us to examine the expression patterns of *CYP39A1* and *CYP7B1*, enzymes involved in the metabolism of these two oxyterols. *In vivo*, 27OHC can be metabolized by CYP7B1 to 7α-hydroxy-3-oxo-4-cholestenoic acid and can also be further metabolized by CYP27A1 to 3β-hydroxy-5-cholestenoic acid (Bjorkhem *et al.* 2009). CYP39A1 which is also expressed in the brain (Shafaati *et al.* 2009) is the enzyme responsible for metabolizing 24OHC (Li-Hawkins *et al.* 2000). The observed expression profile of CYP7B1 has a parallel profile to CYP27A1, however little is known about the transcription regulation of CYP7B1. Nevertheless, there is a significant increase in CYP7B1 in NT2N cells that may explain the observed decrease in 27OHC levels. CYP39A1 levels are also significantly increased in post-mitotic neurons, which may

account for the lack of enzyme-product correlation observed for CYP46A1. Indeed, it has been suggested that induction of CYP39A1 may lead to the production of 7α-24OHC and mask any increase in 24OHC content (Shafaati *et al.* 2009).

Alternatively, CYP46A1 has been shown to further metabolize 24OHC into 24,27-dihydroxycholesterol, binding to 24OHC with an higher affinity than to cholesterol (Mast *et al.* 2003). Therefore, such an effect would mask any increase in the production of 24OHC, and it would be extremely relevant to quantify 24,27-dihydroxycholesterol in NT2N cells. Furthermore, the fact that the decrease in 27OHC occurs concomitantly with the increase in CYP46A1, and due to the broad substrate specificity of CYP46A1 (Mast *et al.* 2003), it is feasible that, not only 24OHC is converted to the 24,27-dihydroxycholesterol, but also that 27OHC in excess could be further metabolized by the highly expressed CYP46A1 in post-mitotic neurons. Due to its physiologic relevance, we are currently investigating the hypothesis that 27OHC can be a substrate for CYP46A1.

Herein we show that NT2N post-mitotic neurons express several genes involved in brain cholesterol synthesis and synthesis regulation, uptake and catabolism. Furthermore, this is the first time CYP46A1 protein and 24OHC have been detected in human neuronal cells in culture. More importantly, our results indicate that during neurogenesis there seems to be a shift in the cholesterol elimination pathways, from a 27-hydroxylation-dependent pathway to a 24-hydroxylation-dependent pathway. Taken together, our results suggest differentiated NT2 as a good experimental cell system for the study of cholesterol homeostasis in the human neurons.

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CHROMATIN-MODIFYING AGENTS INCREASE TRANSCRIPTION OF *CYP46A1*, A KEY PLAYER IN BRAIN CHOLESTEROL ELIMINATION

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5.1 Abstract

The major mechanism of brain cholesterol elimination is the conversion of cholesterol into 24(*S*)-hydroxycholesterol by CYP46A1, a neuron-specific cytochrome P450. Since increasing evidence suggests that upregulation of *CYP46A1* may be relevant for the treatment of Alzheimer's disease, we aim to identify the molecular mechanisms involved in *CYP46A1* transcription. Our previous studies demonstrated the role of Sp transcription factors in basal expression and histone deacetylase (HDAC) inhibitor-dependent derepression of *CYP46A1*. Here, we show that the demethylating agent 5'-Aza-2'-deoxycytidine (DAC) is a *CYP46A1* inducer and that pre-treatment with DAC causes a marked synergistic activation of *CYP46A1* transcription by trichostatin A. Surprisingly, bisulfite sequencing analysis revealed that the *CYP46A1* core promoter is completely unmethylated in both human brain and non-neuronal human tissues where CYP46A1 is not expressed. Therefore, we have investigated Sp expression levels by western blot and real-time PCR, and their binding patterns to the *CYP46A1* promoter, by electrophoretic mobility shift assay and chromatin immunoprecipitation assays, after DAC treatment. Our results showed that DAC decreases not only Sp1 and Sp3 protein levels, but also the binding activity of Sp3 to the +1 region of the *CYP46A1 locus*. Concomitantly, HDAC1 and HDAC2 were also significantly dissociated from the promoter. In conclusion, DAC induces *CYP46A1* gene expression, in a DNA methylation-independent mechanism, decreasing Sp3/HDAC binding to the proximal promoter. Furthermore, by affecting the expression of the Sp3 transcription factor in neuronal cells, DAC might affect not only brain cholesterol metabolism, but also the expression of many other neuronal genes.

5.2 Introduction

DNA methylation and histone modifications are major epigenetic events that regulate gene expression. Therefore, in recent years, emphasis has been put on the clinical application of epigenetic drugs, namely DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors. Despite the substantial evidence on the effect of these drugs on the regulation of tumor suppressor genes, little is known about how the cross-talk between DNA methylation and histone acetylation affects transcription of genes involved in the pathophysiology of neurodegenerative disorders.

The cholesterol 24-hydroxylase (CYP46A1) is a cytochrome P450 enzyme, exclusively expressed in neurons (Lund *et al.* 1999), and responsible for the conversion of cholesterol to 24(*S*)-hydroxycholesterol (24OHC). This represents the major pathway of cholesterol elimination from the brain, since this oxysterol can traverse the blood brain barrier, enter circulation, and be cleared by the liver (Russell *et al.* 2009). The importance of CYP46A1 in the normal functioning of the central nervous system (CNS) has been highlighted by studies on *Cyp46a1* knock-out mice. Despite having a normal development when compared to the wild type littermates, *Cyp46a1*$^{-/-}$ mice have a decrease in brain cholesterol synthesis and exhibit severe deficiencies in spatial, associative and motor learning, and hippocampal long-term potentiation (LTP) (Kotti *et al.* 2006).

Due to its importance in the maintenance of brain cholesterol homeostasis, growing evidences suggest that an increase in *CYP46A1* expression could be beneficial in neurodegenerative disorders, especially in Alzheimer's disease (AD). CYP46A1 up-regulation may increase cholesterol clearance, either by increasing cholesterol hydroxylation and 24OHC formation (Wang *et al.* 2008b) or by increasing ABCA1 and *APOE* expression in a liver X receptors (LXR) dependent mechanism (Fukumoto *et al.* 2002, Whitney *et al.* 2002). Moreover, activation of LXR has been reported to attenuate the neuroinflammatory response of primary mixed glial cultures to fibrillar amyloid β (Aβ) (Zelcer *et al.* 2007). Overexpression of *CYP46A1*, or treatment with 24OHC has also been shown to increase the α-secretase activity as well as the α/β-secretase activity ratio (Famer *et al.* 2007, Prasanthi *et al.* 2009). Importantly, a recent study has demonstrated that *in vivo* overexpression of CYP46A1 before or after the onset of amyloid plaques significantly reduces Aβ pathology in mouse models of AD (Hudry *et al.* 2009).

Taken together, these data clearly highlight the importance of understanding the molecular mechanisms that govern human *CYP46A1* expression. Our previous work has demonstrated that human *CYP46A1* promoter reporter constructs present high luciferase activity in cell lines where CYP46A1 mRNA could not be detected and that these recombinants are strongly transactivated *in vitro* not only by the brain-specific Sp4, but also by ubiquitous Sp1 and Sp3 (Milagre *et al.* 2008). These facts suggest that *CYP46A1* gene transcription might be regulated by epigenetic modifications, such as DNA methylation and/or histone modifications. Indeed, our previous studies have shown that inhibition of HDACs activity by trichostatin A (TSA), valproic acid and sodium butyrate causes a potent induction of human *CYP46A1* expression (Nunes *et al.* 2010). Moreover, our results suggested that induction of *CYP46A1* by TSA is correlated with a local shift in Sp1/ Sp3/ Sp4 ratio bound to the *CYP46A1* proximal promoter, which favored the detachment of HDAC1 and HDAC2 as well as the recruitment of histone acetyltransferases and RNA polymerase II (Nunes *et al.* 2010).

The DNMT inhibitor 5'- Aza - 2'- deoxycytidine (DAC) is a potent inhibitor of genomic and promoter methylation, by inhibiting DNMT activity, through irreversible binding of DNMTs to DAC substituted DNA (Creusot *et al.* 1982, Christman *et al.* 1985). It is generally accepted that DAC and TSA synergistically affect gene expression, by a mechanism that depends on promoter demethylation induced by DAC and subsequent reinforcement by histone acetylation induced by TSA. Nevertheless, several studies have suggested that DAC can also affect gene transcription by a mechanism independent of promoter demethylation (Soengas *et al.* 2001, Scott *et al.* 2006).

Here we show that the treatment of SH-SY5Y neuroblastoma cells with DAC enhances TSA-dependent induction of the *CYP46A1* gene, by decreasing Sp3/ HDAC binding to the promoter, in a DNA demethylation-independent manner.

5.3 Methods

5.3.1 Source of human samples

In accordance with the Institutional Medical Ethics Committee, all tissue specimens were collected after patient's informed consent. Human stomach specimens were obtained from individuals who underwent diagnostic stomach endoscopy, but whose results were negative for any stomach-associated pathology (Hospital de Santa Maria,

Lisboa, Portugal). Peripheral blood samples were obtained from healthy volunteers recruited from faculty staff by advertisement and used to prepare lymphocytes. Frozen human brain samples (cortices) were obtained from the Institute of Neuropathology, IDIBELL (Hospital Universitari de Bellvitge, University of Barcelona, Hospitalet de LLobregat, Barcelona, Spain). Those samples were control brains with no neurological abnormalities, cognitively-screened in life and with no neuropathological abnormalities.

5.3.2 Antibodies

The antibodies used in chromatin immunoprecipitation (ChIP), electrophoretic mobility shift assay (EMSA), and western blot are listed in Table 5.1.

Table 5.1. List of the antibodies used in western blot, electrophoretic mobility shift assay (EMSA), andchromatin immunoprecipitation (ChIP).

Protein	Clone/ Reference	Source	Assay
Sp1	39058	Active Motif, Inc. (Rixensart, Belgium)	ChIP
Sp1	PEP-2	Santa Cruz Biotechnology, Inc. (Santa Cruz)	EMSA, western blot
Sp3	D-20	Santa Cruz Biotechnology Inc. (Santa Cruz)	ChIP, EMSA, western blot
Sp4	V-40,	Santa Cruz Biotechnology Inc. (Santa Cruz)	ChIP, EMSA, western blot
HDAC1	05-100	Upstate Biotechnology (Lake Placid, NY, USA)	ChIP, western blot
HDAC2	05-814	Upstate Biotechnology (Lake Placid, NY, USA)	ChIP,western blot
RNA polymerase II	05-623	Upstate Biotechnology (Lake Placid, NY, USA)	ChIP

5.3.3 Cell Culture and treatment

SH-SY5Y (human neuroblastoma) and HeLa (cervix adenocarcinoma) cell lines were maintained as previously described (Milagre *et al.* 2008). The optimal concentration of DAC was determined by performing dose response assays in both cell lines. Subsequently, all cells were treated with 5 μM DAC or/ and 0.25 μM TSA for the indicated time points or with vehicle (water or ethanol, respectively). Cell culture medium was changed every 24 hours.

5.3.4 mRNA analysis

Total RNA from treated or untreated cells was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions. 1.5 μg of RNA was reversed transcribed using SuperScript II reverse-transcriptase kit with random hexamer primers (Invitrogen), and first strand DNA from 150 ng of RNA was used as a template in quantitative real-time PCR (qPCR) with an ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA). The cycling program was set as follows: denature at 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 sec and 60 ºC (for CYP46A1, Sp3 and β-actin) or 63 ºC (for Sp1 and Sp4) for 1 min. The Taqman® gene expression assay and the primers used in this study are described in table 5.2. Results presented were obtained from three individual experiments and each sample was assayed in triplicate. CYP46A1 mRNA levels were normalized to the level of β-actin and expressed as pg of CYP46A1 mRNA per ng of β-actin mRNA. Sp1, Sp3 and Sp4 mRNA levels were normalized to the level of β-actin and expressed as fold change from controls, using the $^{\Delta\Delta}$Ct method. Four candidate genes were analyzed with the Norm Finder algorithm (Andersen *et al.* 2004) and β-actin was found to be the most stable single gene to be used as an endogenous control.

5.3.5 Genomic DNA isolation and bisulfite sequencing analysis

Tissue genomic DNA was isolated using the Puregene Cell and Tissue kit (Gentra Systems, Minneapolis, MN, USA). Genomic DNA from SH-SY5Y cells was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. A PCR product encompassing the proximal promoter region was amplified from genomic DNA, *in vitro* methylated with *SssI* methylase or mock methylated and used as methylated or unmethylated control, correspondingly. Bisulfite sequencing analysis was performed using 1μg of genomic DNA with the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA).

Bisulfite modified DNA was PCR amplified using two pairs of primers that covered a 557bp region of *CYP46A1* proximal promoter containing the 438bp CpG island (Table 5.2), amplifying the +1 to +177 and the -380 to +21 region of the CYP46A1 promoter. The PCR products were then cloned into the pCR®2.1 vector (Invitrogen) and 24 colonies of each reaction were purified and analyzed by sequencing.

5.3.6 Nuclear Extracts

Nuclear extracts were prepared as described by Schreiber and co-workers (Schreiber *et al.* 1989), subjected to 7.5% SDS-PAGE gels and electroblotted onto Immobilon P (Millipore, Bedford, MA, USA). After visualization of the transferred proteins by amido black staining, the membranes were incubated with specific antibodies. Results were quantified using the Quantity One densitometry analysis software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Table 5.2. Sequence of primers used in this study.

Gene	Primer / TaqMan Assay	Sequence (5'→ 3') / Source	Assay
CYP46A1	CYP46A1 HS00198510_M1	Applied Biosystems	qPCR
β Actin	ACTB HS99999_M1	Applied Biosystems	qPCR
Sp1	qPCRSp1fwd qPCRSp1Rev	5'-gcctccagaccattaacctcagt -3' 5'- gctccatgatcacctggggcat -3'	qPCR
Sp3	Sp3qPCRfwd2 Sp3qPCRRev2	5'-gctggtatacagctacatccaggag -3' 5'-caccactgagctgccactcttc -3'	qPCR
Sp4	qPCRSP4Fwd qPCRSP4Rev	5'- gcagcggcgatggctacaga -3' 5'- agggctgagagtcctgggagc -3'	qPCR
β Actin	qPCRHuACTINf qPCRHuACTINr	5'-ctggaacggtgaaggtgaca-3' 5'-aagggacttcctgtaacaatcca-3'	qPCR
CYP46A1 (+1 to +177)	bCYP46p1F bCYP46p1R	5′-gatttgagtttgaagaggtgg -3′ 5′-caacaacaaccc(g/a)aaactcataac-3′	Bisulfite sequencing
CYP46A1 (-380 to +21)	bCYP46p2F bCYP46p2R	5'- ggagttatgagttt(c/t)gggttgttg -3' 5'-tatccccaaaccccccaatccc-3'	Bisulfite sequencing

5.3.7 Electroforetic Mobility Shift Assay

EMSA was performed as previously described (Milagre *et al.* 2008). Briefly, 2-5 µg NE were incubated with γ32P-labeled oligonucleotide probe harboring sites SP-RE-B or SP-RE-D. For supershift assays, 1 µl of each specific antibody were pre-incubated with the NE for 30 min before the addition of probe. Protein-DNA complexes were resolved on 5 % native polyacrylamide gels and visualized by autoradiography.

5.3.8 Chromatin immunoprecipitation

ChIP assays were performed as previously described in Chapter 3 (Section 3.3.6). The recovered DNA was analyzed by real-time PCR with SYBR green Master Mix in an ABI 7300 sequence detection system (Applied Biosystems). Real-time PCR was performed using primers that covered different regions of the promoter as previously described (Nunes *et al.* 2010).

5.3.9 Statistical analysis

Statistical analysis was performed using the Student's t-test and the ANOVA one-way test with the Tukey HSD post-hoc test or the Tukey HSD for unequal n (Spjotvoll/Stoline test). All analysis was performed using the STATISTICA (data analysis software system), version 7.1 StatSoft, Inc. (2006). A value of $p < 0.05$ was considered significant.

5.4 Results

5.4.1 DAC activates the transcription of human *CYP46A1* gene and acts snergistically with TSA

In our previous studies we have characterized the molecular mechanisms involved in the *CYP46A1* response to HDAC inhibitors (Nunes *et al.* 2010). In this study, we aimed to elucidate if another epigenetic mechanism, namely DNA methylation, is involved in the tissue specific expression of the human *CYP46A1* gene. With this purpose, we investigated the effect of the demethylating agent DAC on the expression of the human *CYP46A1* gene. SH-SY5Y and HeLa cells were treated with increasing concentrations of DAC for 96 h or with 5 μM DAC for increasing periods of time, and *CYP46A1* expression was quantified by real-time PCR (qPCR) (Figure 5.1). The kinetics of CYP46A1 mRNA accumulation in SH-SY5Y treated with 5 μM DAC showed that mRNA levels started to increase significantly 72 h after DAC treatment (ANOVA one-way test: $F = 21.29$, $df = 3$, $p < 0.001$; Tukey HSD for unequal N, $p < 0.01$), reaching a maximum 96 h after treatment (Tukey HSD for unequal N, $p < 0.001$) (Figure 5.1 A). Moreover, treatment with 2.5 μM or 5 μM DAC for 96 h significantly increased the

CYP46A1 mRNA levels, when compared with untreated cells (ANOVA one-way test: F = 10.89, df = 3, $p < 0.01$; Tukey HSD for unequal N, $p < 0.01$) (Figure 5.1 B). In contrast, in HeLa cells treated with 5 μM DAC for 96 h, CYP46A1 mRNA levels remained undetected (data not shown).

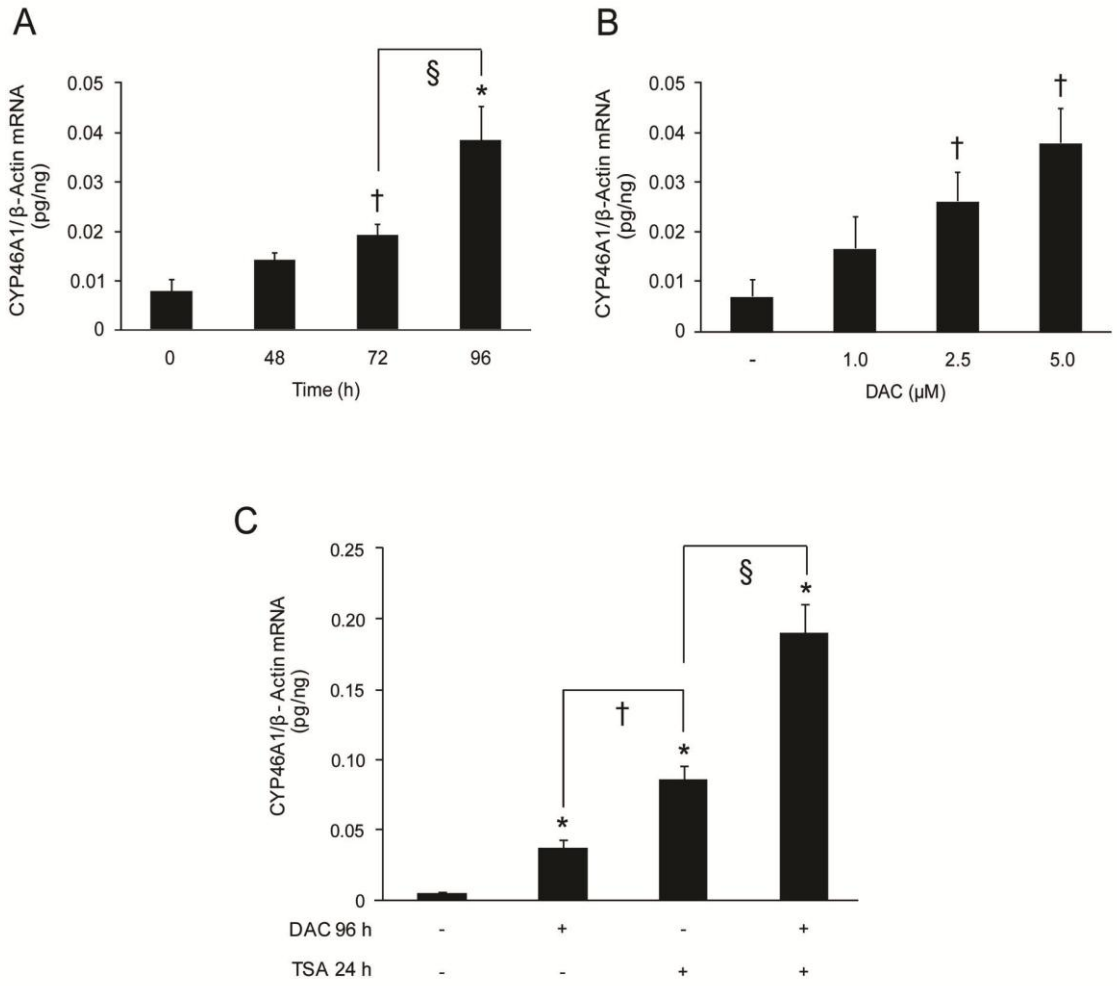

Figure 5.1. DAC increases CYP46A1 mRNA levels and potentiates the TSA-dependent induction of CYP46A1 expression in SH-SY5Y cells. Real-time PCR analysis of CYP46A1 steady-state mRNA transcript levels in SH-SY5Y cells treated with 5 μM DAC for the indicated time points (A), with increasing concentrations of DAC for 96 hours (B), and with 5 μM DAC for 96 hours, 0.25 μM TSA for 16 h, or with both (C). Values were normalized to the internal standard β-actin. Data represent means ± SEM of at least three independent experiments and were expressed as pg of CYP46A1 mRNA per ng of β-actin mRNA (§$p < 0.05$; †$p < 0.01$; *$p < 0.001$).

Since we have previously shown that TSA leads to an up-regulation of *CYP46A1* expression in SH-SY5Y cells (Nunes *et al.* 2010), the coordinated effects of the

demethylating agent, DAC, with the HDAC inhibitor, TSA, were further evaluated (Fig. 1C). Pre-treatment with the DNA demethylating agent caused marked synergistic activation of the *CYP46A1* gene by TSA, resulting in a significant increase of the CYP46A1 mRNA levels when compared with DAC or TSA treated cells (Fig 1B) (ANOVA one-way test: F = 154.1, df = 3, $p < 0.001$; Tukey HSD for unequal N, $p < 0.001$ and $p < 0.05$, respectively).These results demonstrate that *CYP46A1* gene transcription was significantly induced by DAC treatment in SH-SY5Y cells, and that pre-treatment with this DNA demethylating agent caused marked synergistic activation of the *CYP46A1* gene by TSA.

5.4.2 Methylation status of the *CYP46A1* promoter in SH-SY5Y cells and different human tissues

Since DAC led to the derepression of *CYP46A1* expression in SH-SY5Y, we evaluated the methylation status of the proximal promoter in this cell line as well as in human brain, lymphocytes and stomach samples. Analysis of the 5' flanking region of the *CYP46A1* gene (-0.3 kb) and its first exon by a CpG island searcher program (EMBOSS CpGPlot) indicated that the proximal promoter region of this gene is located in a predicted CpG island that encompasses nucleotides -300 to +138 relative to the ATG start codon (+1). This island (all values are observed/expected) was defined based on its size (438bp/ >200bp), GC content (79%/ 50%) and CpG dinucleotide frequency (0,81/ >0,6) (Gardiner-Garden & Frommer 1987). The DNA methylation status of the *CYP46A1* promoter region was assessed by bisulfite sequencing analysis and surprisingly, our results showed that the *CYP46A1* promoter region was completely unmethylated not only in human brain, but also in SH-SY5Y cells and human tissues where *CYP46A1* is not expressed (Figure 5.2). As positive controls, we used an *in vitro* methylated or unmethylated PCR product amplified from genomic DNA, corresponding to the *CYP46A1* proximal promoter region (Figure 5.2). In parallel, we have also assessed the methylation status of the pyruvate dehydrogenase 2 gene promoter, which we have previously shown to be methylated in somatic tissues (data not shown) (Pinheiro *et al.* 2010).These data demonstrated that, as expected, the *CYP46A1* promoter is completely unmethylated in the human brain, where *CYP46A1* is highly expressed.

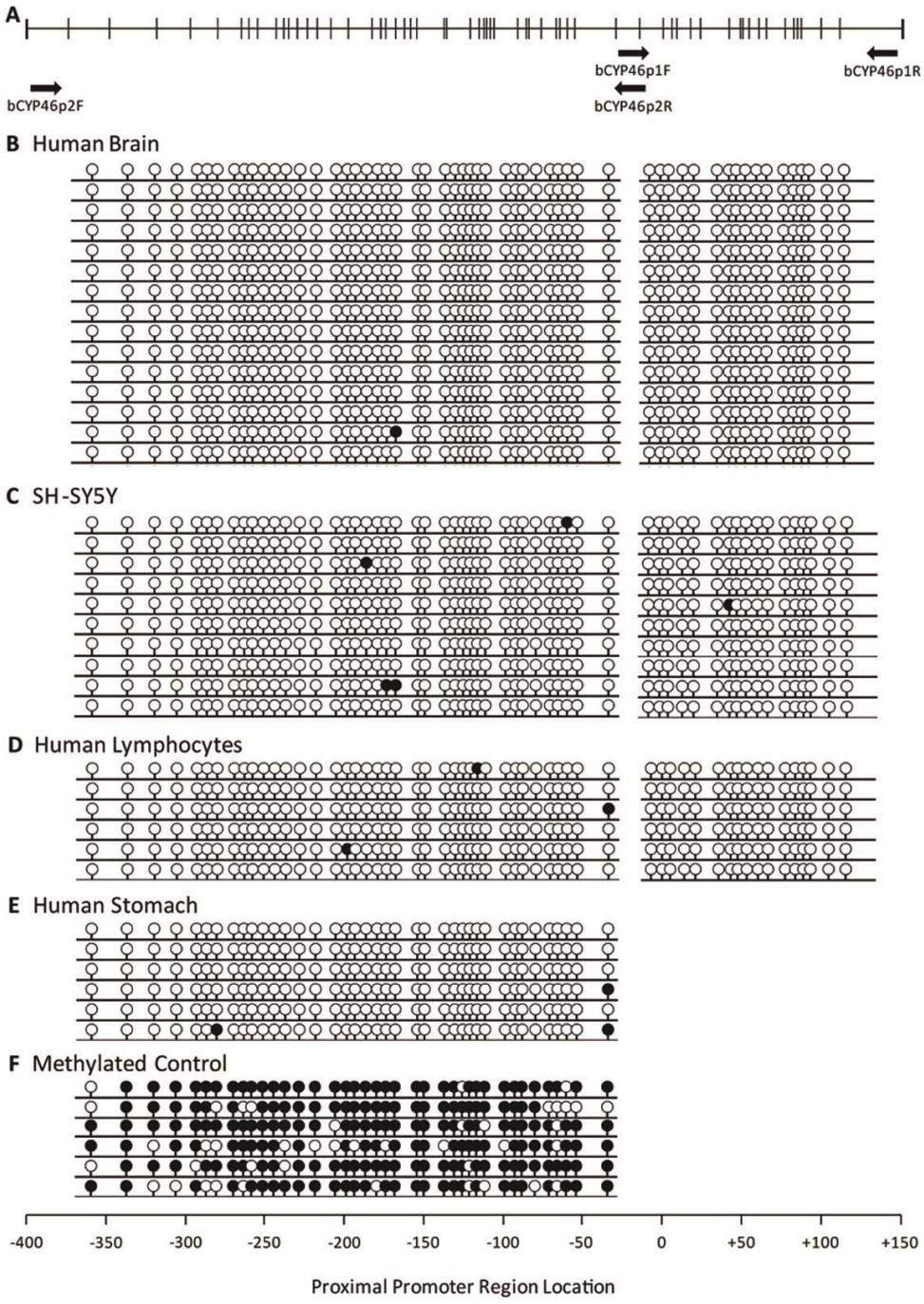

Figure 5.2. Bisulfite sequencing of *CYP46A1* promoter region in SH-SY5Y cells and different human tissues. Bisulfite-treated DNA was PCR amplified using primers spanning the CpG island containing the proximal promoter region of *CYP46A1*. PCR products were cloned, and multiple clones were sequenced and analyzed for their methylation status. A) Map of *CYP46A1* proximal promoter region showing location of CpG sites (vertical dashes) and primers used for bisulfite sequencing (arrows). Bisulfite sequencing results are shown for human brain (B), SH-SY5Y cells (C), human lymphocytes (D), human stomach (E), and *in vitro* methylated DNA as a control (F). Open and filled circles indicate unmethylated and methylated CpG sites, respectively. The CpG sites are mapped in reference to the translation start site.

Surprisingly, our data also showed that the same region is also completely unmethylated in human tissues where CYP46A1 mRNA cannot be detected, suggesting that DNA methylation of the proximal promoter does not play a role in the regulation of *CYP46A1* tissue specific expression. Interestingly, the *CYP46A1* gene responded to DAC treatment, in SH-SY5Y cells, even though the promoter is already completely unmethylated in these cells. However, we cannot exclude that methylation might be affecting an enhancer or other regulatory element not yet identified, outside of the *CYP46A1* core promoter.

5.4.3 Treatment with the demethylating agent DAC causes a shift in the Sp protein levels ratio

The fact that *CYP46A1* was induced by DAC treatment in SH-SY5Y cells, even though the proximal promoter was completely unmethylated, led us to investigate if the expression levels of the Sp transcription factors, previously described as key regulators of *CYP46A1* (Milagre *et al.* 2008), were being affected by this drug. In fact, 72 h after treatment with 5 μM DAC, we could observe a significant decrease in the protein levels of Sp1 and the different Sp3 isoforms (ANOVA one-way test: $F = 14.8$, $df = 4$, $p < 0.001$; Tukey HSD for unequal N, $p < 0.05$; and ANOVA one-way test: $F = 20.62$, $df = 4$, $p < 0.001$; Tukey HSD for unequal N, $p < 0.01$, respectively) (Figure 5.3 A and B). The levels of both Sp1 and Sp3 remained low 96 h after treatment. In contrast, the brain specific Sp4 protein levels were not affected upon treatment with 5 μM DAC.

Interestingly, in HeLa cells where DAC treatment did not affect *CYP46A1* expression, the levels of Sp1, Sp3 and Sp4 protein were also not affected by this DNA demethylating agent (Figure 5.3A). To further understand the mechanisms by which DAC treatment was affecting the Sp expression levels, we assessed the mRNA levels of the different Sp transcription factors by real-time PCR, after DAC treatment. Our results showed that DAC treatment did not significantly alter the mRNA levels of Sp1, Sp3 or Sp4 (Figure 5.3 C).

Our results showed that Sp1 and Sp3 protein levels were significantly decreased by treatment with DAC and this decrease was concomitant with the increase in CYP46A1 mRNA levels.

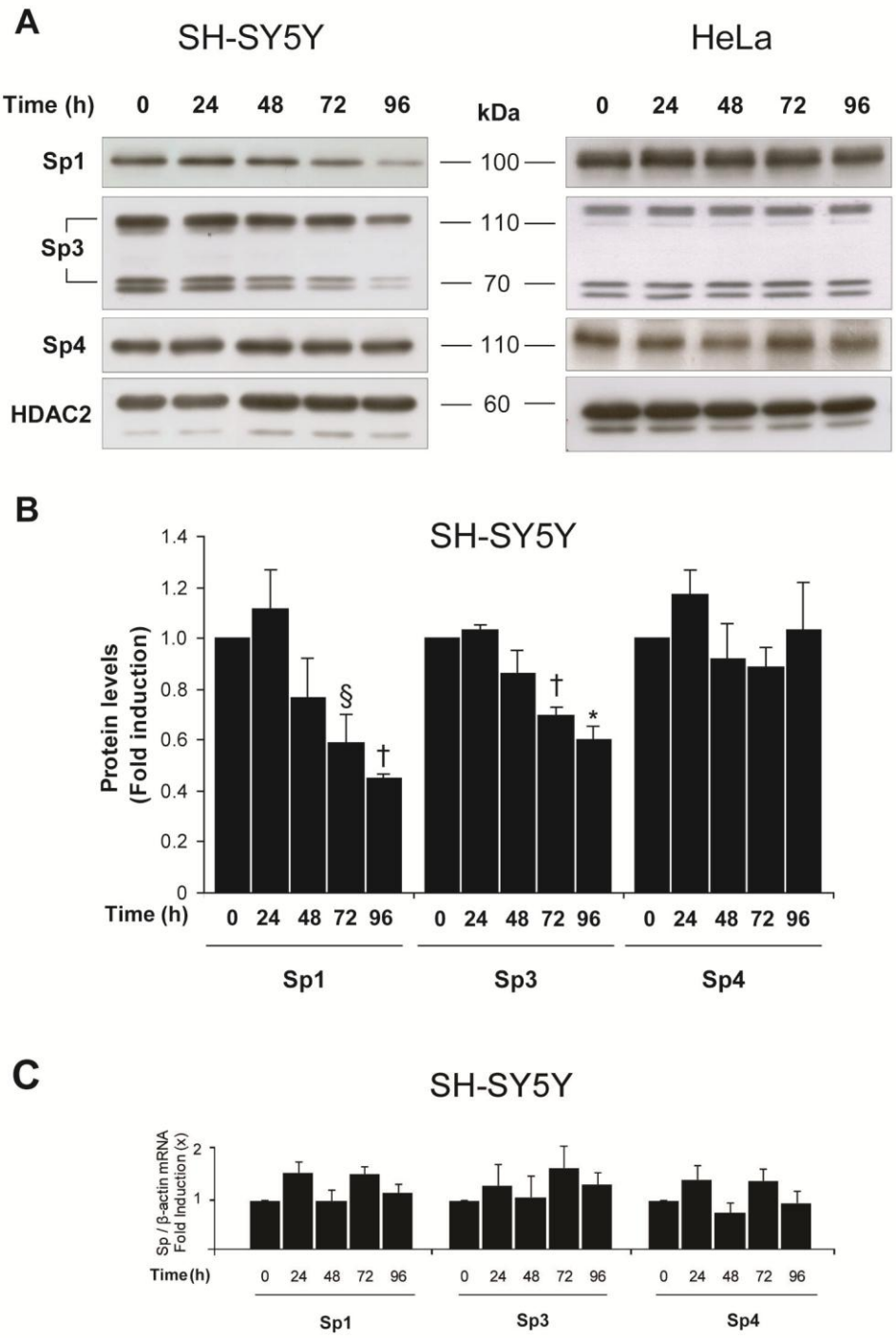

Figure 5.3. Treatment with DAC affects Sp transcription factors protein levels. SH-SY5Y or HeLa cells were treated with 5 μM DAC for the indicated time points. A and B) Nuclear extracts were subjected to SDS-PAGE, transferred to PVDF membranes, and the membranes were incubated with specific anti -Sp1, -Sp3 and -Sp4 antibodies. The HDAC 2 levels were determined as a loading control. C) Real-time PCR analysis of Sp1, Sp3 and Sp4 steady-state mRNA transcript levels. Values were normalized to the internal standard β-actin. All results shown are representative of those obtained in at least three independent cultures. Data represent means ± SEM and are expressed as fold change relative to control cells (§$p < 0.05$; †$p < 0.01$; *$p < 0.001$).

5.4.4 Recruitment of Sp proteins to the *CYP46A1* promoter after treatment with DAC alone or in combination with TSA

Afterwards, we have investigated whether the decrease in Sp1 and Sp3 protein levels was correlated with changes in the different Sp protein binding activities to the Sp response elements in the *CYP46A1* proximal promoter, or to the formation of novel DNA-protein complex(es) after DAC treatment. We have incubated nuclear proteins isolated from SH-SY5Y cells treated with 5 μM DAC for different time points, with the Sp-RE-B and Sp-RE-D probes, which we had already shown to have a high affinity for the different Sp proteins (Milagre *et al.* 2008). The EMSA results showed a time-dependent decrease in the total nuclear proteins capable of binding to the Sp-RE-B probe after DAC treatment (Figure 5.4 A). Similar results were obtained with the Sp-RE-D probe (data not shown). In agreement with our previous results (Nunes *et al.* 2010), Sp3 was the major component of these protein complexes, and the composition of the complexes formed with nuclear extracts prepared from cells treated with 5 μM DAC for 72 h did not greatly differ from those formed with nuclear extracts prepared from control cells (Figure 5.4 B).

Since we have previously demonstrated that the total nuclear proteins capable of binding to the Sp-RE-B probe decreased after TSA treatment (Nunes *et al.* 2010), we decided to analyze the effect of treatment with 5 μM DAC for 72 h, 0.25 μM TSA for 6 hours, or the combined treatment, in the binding activities of Sp proteins to the Sp-RE-B element. As expected, TSA treatment led to a decrease in total nuclear proteins capable of binding to the probe, when compared with the vehicle-treated cells (Figure 5.4 C). Interestingly, when we used nuclear proteins prepared from cells treated with DAC alone or in combination with TSA, we could observe a decrease in Sp protein binding to the Sp-RE-B element when compared with TSA-treated cells. Nevertheless, treatment with both the DNA demethylating agent and the HDAC inhibitor does not elicit a further decrease in Sp proteins binding affinity, when compared with DAC alone (Figure 5.4 C).

These results show that DAC not only decreases Sp1 and Sp3 levels but also decreases the binding activity of these transcription factors to the Sp-RE elements in the *CYP46A1* proximal promoter.

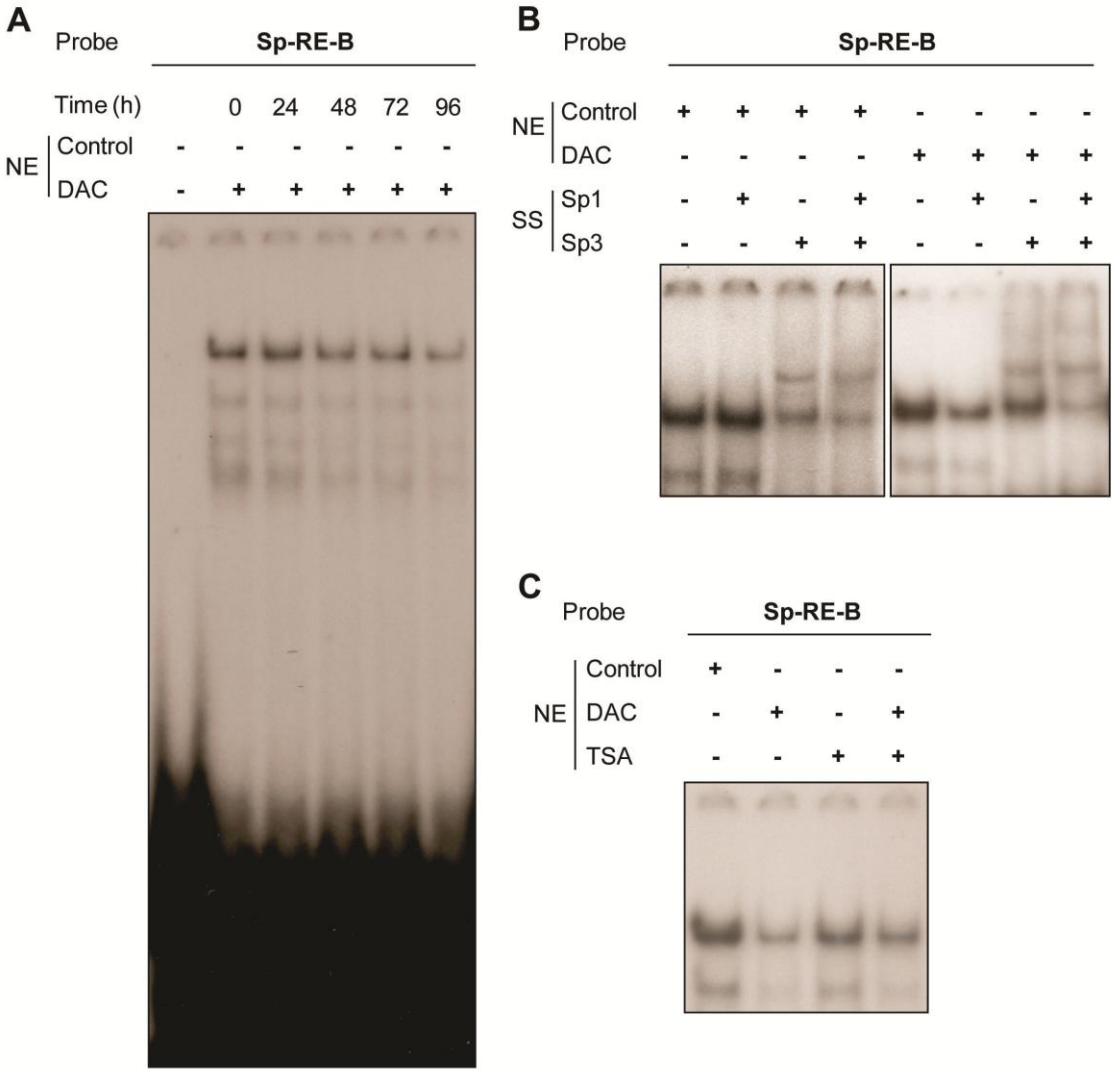

Figure 5.4. DAC affects Sp protein binding to the SP-RE-B site of the CYP46A1 proximal promoter in human neuroblastoma cells. EMSA were performed using nuclear extracts (NE) of SH-SY5Y cells and a radiolabeled double-stranded oligonucleotide corresponding to the Sp-RE-B site as a probe. A) The probe was incubated with NE prepared from cells treated with vehicle or with 5 µM DAC for the indicated time points. B) Super-shift (SS) experiments were performed using NE of cells treated with vehicle or with 5 µM DAC for 72 hours, and anti-Sp1 and -Sp3 antibodies. C) The probe was incubated with NE prepared from cells treated with vehicle or with 5 µM DAC for 72 hours, 0.25 µM TSA for 6 hours or both. Autoradiograms are representative of three independent experiments.

To further understand if the multiprotein complexes bound to the proximal promoter were being affected by the treatment with DAC alone or in combination with TSA, we performed ChIP assays. We have previously shown that in untreated and TSA-treated

SH-SY5Y cells, both the ubiquitous Sp1 and Sp3 as well as the brain enriched Sp4 were present in the *CYP46A1* +1 region of the promoter (Nunes *et al.* 2010). Since our results show that Sp protein levels were affected by DAC treatment, the binding of these proteins to the promoter region was determined (Figure 5.5). Confirming our previous results, in untreated SH-SY5Y cells, Sp1, Sp3 and Sp4 were present in the +1 region (Figure 5.5 A and B). In agreement with our EMSA results, we observed a significant decrease in the binding of the Sp3 transcription factor to the proximal promoter, 72 h after treatment with 5 μM DAC (ANOVA one-way test: $F = 5.50$, $df = 3$, $p < 0.01$; Tukey HSD for unequal N, $p < 0.01$). DAC treatment of SH-SY5Y cells did not affect the binding of any of the other Sp transcription factors to the GC-rich proximal promoter region of the *CYP46A1* gene (Figure 5.5 B), suggesting that, although DAC induces a decrease in Sp1 levels, this decrease is not reflected in the binding of this transcription factor at the promoter level. We further examined the binding of HDACs to the *CYP46A1* promoter. HDAC1 and HDAC2 were significantly dissociated from the +1 promoter region, 72 h after DAC treatment, since there were no longer significant differences between the chromatin recovered with the anti-HDAC1 and 2 antibodies, and that recovered with IgG (Figure 5.5 B).

We further analyzed the recruitment of Sp3 and RNA polymerase II (RNA pol II) to the *CYP46A1* promoter in cells treated with either TSA or with the combined treatment of DAC and TSA. As previously demonstrated, TSA did not induce an overall decrease in Sp3 binding to the +1 region (Nunes *et al.* 2010) but in cells treated with DAC and TSA the Sp3 association to the proximal promoter was diminished by approximately 50%, a decrease similar to the one obtained after DAC treatment (Figure 5.6).

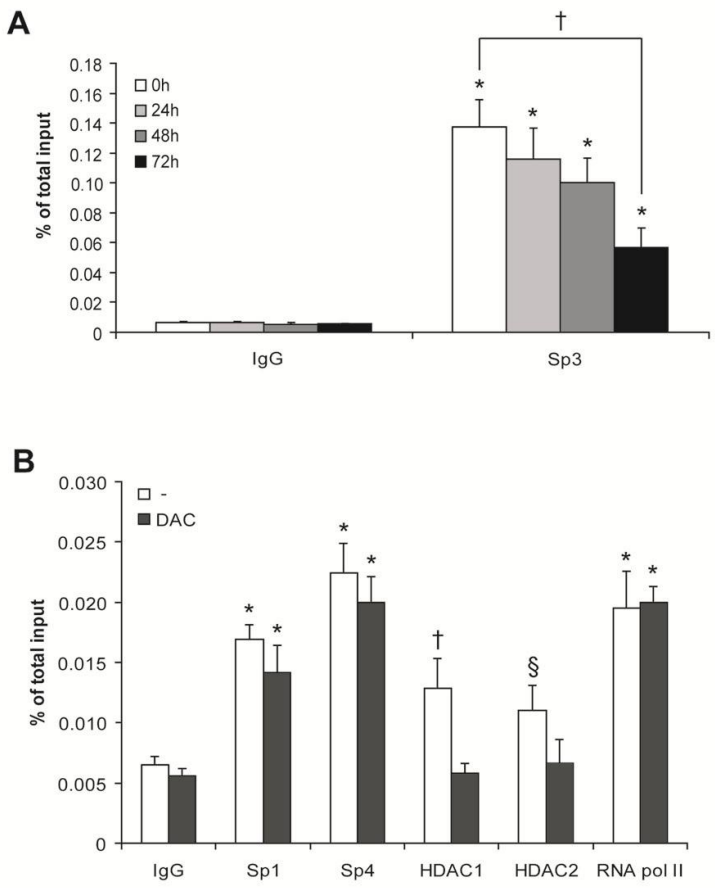

Figure 5.5. Decrease of Sp3 and HDAC binding to the CYP46A1 proximal promoter after DAC treatment. Chromatin from SH-SY5Y cells treated with 5 µM DAC for the indicated time points (A), or for 72h (B) was prepared and immunoprecipitated with anti-Sp1, -Sp3, -Sp4, -HDAC1, -HDAC2 and -RNA pol II antibodies. After DNA recovery, the precipitates were evaluated by real-time PCR. All results are expressed as percentage of total input and represent means ± SEM of at least three independent experiments (§$p < 0.05$; †$p < 0.01$; *$p < 0.001$).

These results are in agreement with our EMSA results, where treatment with both the DNA demethylating agent and the HDAC inhibitor did not elicit a further decrease in Sp proteins binding affinity, when compared with DAC alone (Figure 5.4). Furthermore, we were expecting to detect a correlation between recruitment of RNA pol II and CYP46A1 mRNA levels. Nevertheless, although we have confirmed our previous results, showing a significant recruitment of RNA pol II after TSA treatment, DAC alone did not alter RNA pol II association to the proximal promoter, and pre-treatment with DAC did not further enhance the TSA-dependent recruitment.

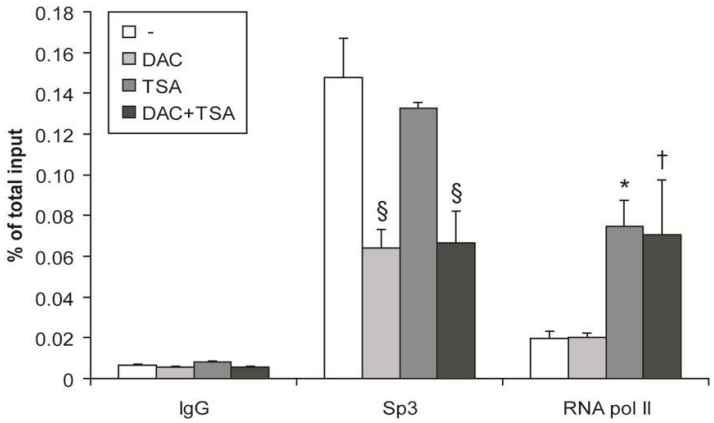

Figure 5.6. Recruitment of Sp3 and RNA pol II to the GC-rich region of the CYP46A1 proximal promoter, after combined treatment with DAC and TSA. Chromatin from SH-SY5Y cells treated with 5 μM DAC for 72h, 0.25 μM TSA for 6 hours or both, was prepared and immunoprecipitated with anti-Sp3 and -RNA pol II antibodies. After DNA recovery, the precipitates were evaluated by real-time PCR. All results are expressed as percentage of total input and represent means ± SEM of at least three independent experiments (§$p < 0.05$; †$p < 0.01$; *$p < 0.001$, relative to vehicle treated cells).

5.5 Discussion

CYP46A1 is a key player in the maintenance of cholesterol homeostasis in the brain. Increasing evidence suggests that upregulation of CYP46A1 may benefit AD treatment. Indeed, injection of a adeno-associated vector encoding *CYP46A1* in the brain of AD transgenic mice, before or after the onset of amyloid deposits, can significantly reduce Aβ pathology *in vivo* (Hudry *et al.* 2009). Furthermore, *in vitro* overexpression of CYP46A1, or treatment with 24OHC increases α-secretase activity as well as α/β-secretase activity ratio (Famer *et al.* 2007, Prasanthi *et al.* 2009).

HDAC inhibitors are the only compounds known to up-regulate the expression of *CYP46A1* gene (Shafaati *et al.* 2009, Nunes *et al.* 2010). In the present study, we have identified DAC, a Food and Drug Administration approved drug that effectively passes the blood brain barrier (Chabot *et al.* 1983), as an inducer of the *CYP46A1* gene and characterized the molecular mechanisms involved in the potentiation of TSA-dependent *CYP46A1* induction by this DNMT inhibitor. To explore the involvement of DNA methylation and histone modifications in *CYP46A1* gene silencing, we first treated different cell lines with DAC and TSA. Synergistic derepression of the *CYP46A1* gene by DAC and TSA or derepression with DAC alone was observed in SH-SY5Y neuroblastoma cells, but was absent in HeLa cells. In addition, we investigated the DNA

methylation profile of the CpG island that contains the *CYP46A1* proximal promoter region, covering the Sp-binding sites previously shown to be important for *CYP46A1* basal expression. Surprisingly, the bisulfite sequencing analysis revealed that the *CYP46A1* core promoter is completely unmethylated, even in human tissues where CYP46A1 is not expressed, which suggests that DNA methylation of the proximal promoter does not play a role in the regulation of *CYP46A1* tissue specific expression. We have previously demonstrated that the effect of TSA on CYP46A1 mRNA levels was virtually instantaneous, detectable by immediate localized histone hyperacetylation (Nunes *et al.* 2010), which is in favor of an unmethylated promoter. Moreover, by ChIP analysis we could not detect any association of DNMT1 or MeCP2 to the *CYP46A1* promoter in untreated SH-SY5Y cells (data not shown). Nevertheless, we cannot exclude that DNA demethylation may reactivate an unidentified transcription factor that controls *CYP46A1* expression, or that methylation of an enhancer or other regulatory element not yet identified, outside of the *CYP46A1* core promoter, can interfere with its expression.

The mechanism underlying DAC-mediated relieve of *CYP46A1* repression in neuroblastoma cells is not clearly known. DAC had been previously reported to induce the expression of unmethylated silenced genes (Zhu *et al.* 2001, Soengas *et al.* 2001, Scott *et al.* 2006). Interestingly, two of these genes $p19^{INK4d}$ and $p21^{WAF1/Cip}$ are also Sp-regulated genes (Yokota *et al.* 2004, Kim *et al.* 2003). Therefore, we have investigated Sp expression levels, and binding patterns to the *CYP46A1* promoter, by western blot, real-time PCR, EMSA and ChIP analysis, after DAC treatment. DAC treatment decreased not only Sp1 and Sp3 protein levels, but also the binding activity of Sp3 to the +1 region of the *CYP46A1 locus*. An effect of DAC and 5-aza cytidine on the expression levels of Sp proteins had been previously reported. Indeed, treatment of MCF-7L breast and GEO colon cancer cells with 5-aza cytidine led to down-regulation of Sp3, but up-regulation of Sp1 (Ammanamanchi & Brattain 2001, Periyasamy *et al.* 2000). Interestingly, in HeLa cells, where CYP46A1 mRNA levels were not induced by DAC, Sp protein levels were also not affected. Our results also revealed that, although affecting protein levels of both Sp1 and Sp3, DAC treatment did not alter mRNA levels of the different Sp transcription factors. Interestingly, DAC affects levels of specific proteins by rapidly inducing degradation by the proteasomal pathway (Cecconi *et al.* 2009, Patel *et al.* 2010).

Concomitant with the decrease in Sp3 binding to the *CYP46A1* +1 region, ChIP experiments also revealed that HDAC1 and HDAC2 were significantly dissociated from the promoter. DAC had already been reported to relieve $p21^{WAF1}$ repression in acute

myeloid leukemia cells by a mechanism that involved release of HDAC1 (Scott *et al.* 2006). The decrease in Sp3 binding induced by DAC is in agreement with our previous results, which showed that the decrease of Sp3 at particular responsive elements induced by TSA, namely at the Sp-RE-B site, could locally shift the Sp1/ Sp3/ Sp4 ratio, and favor the detachment of HDAC1 and HDAC2 from the *CYP46A1* promoter (Nunes *et al.* 2010). Sp3 has been demonstrated to physically interact with specific repressor complexes, namely with HDACs and mSin3A (Zhang & Dufau 2002, Clem & Clark 2006, Sun *et al.* 2002). Loss of Sp3 binding induced by DAC, which might be required for HDAC association, may facilitate TSA-induced acetylation of the histone tail and the maintenance of the open configuration of the local chromatin, suggesting that this drug can directly induce regional chromatin remodeling, independently of its effects on DNA methylation. Although DAC treatment did not induce an enrichment of RNA pol II at the *CYP46A1* promoter, as we would expect, we can hypothesize that in a HDAC-free promoter, recruitment of basal transcriptional machinery is facilitated. It would have been interesting to monitor by ChIP, phosphorylation of specific serine residues in the carboxy-terminal domain to further assess initiating, productive and elongating RNA pol II.

The use of drugs that modify the epigenome has been recently highlighted in brain disorders, namely in AD. In the Tg2576 mouse model of AD, 4-phenylbutyrate reversed spatial memory deficits by normalizing Tau hyperphosphorylation in the hippocampus, without affecting Aβ levels (Ricobaraza *et al.* 2009). Moreover, in the AβPP23 transgenic mouse model of AD, low doses of valproic acid significantly reduced Aβ plaque number and improved memory deficits (Qing *et al.* 2008). The importance of histone acetylation for synaptic plasticity and memory formation has also been demonstrated. Environmental enrichment caused chromatin modification through increased histone-tail acetylation, and reinstated learning and memory even after significant neurodegeneration had taken place in CK-p25 transgenic mice (Fischer *et al.* 2007). In a follow-up study, mice overexpressing HDAC2, but not HDAC1, exhibited decreased dendritic spine density, synaptic number and synaptic plasticity, and impaired memory formation (Guan *et al.* 2009). Conversely, *Hdac2* knockout mice showed memory improvement, while the symptoms induced by HDAC2 overexpression, were partially reverted by treatment with the HDAC inhibitor vorinostat.

Nevertheless, some caveat is necessary when exploring the potential use of epigenetic drugs in neurodegeneration. First of all, it has to be considered that the

development of neurodegenerative diseases is often very slow and the onset not precisely defined. The side-effects related to the use of epigenetic drugs for long periods of time that cover the period of Mild Cognitive Impairment, is not yet known. One of the major concerns deals with the possible negative effect that epigenetic drugs could exert on cancer. It is largely known that changes in methylation patterns of oncogenes and oncosuppressor genes are associated to cancer onset and development. For this reason, the use of compounds potentially able to modulate the epigenome should be tested, considering the possible altered regulation of a wide number of genes. Wide-analysis approaches like ChIP-arrays, RNA micro-arrays and proteomics assays could represent the obvious road to disclose potential "side-effects" of epigenetic drugs. In this regard, we have already the indication that epigenetic modulation does not necessarily involve the whole genome. In fact, the use of a macro-array representative of about 600 genes expressed in CNS has shown that only 1-2% of such genes were modulated by altered DNA methylation status (Cavallaro *et al.* 2006). A second aspect that should be considered deals with the efficacy of DAC administration and *CYP46A1* induction versus different AD features, like Aβ production and aggregation, and cognitive impairment. To this purpose, the use of cell cultures and AD transgenic animal models will be necessary, but the findings presented in this paper help to elucidate the mechanisms underlying the effects of epigenetic regulation on cholesterol metabolism and represent a platform for the development of a possible epigenetic intervention in AD.

In summary, the fact that DAC has been approved by the Food and Drug Administration to treat myelodysplastic syndrome, that it effectively passes the BBB, and in light of the recent interest on the use of HDAC inhibitors as a therapeutic avenue for neurodegenerative disorders, it is of the utmost importance to characterize the molecular mechanisms by which these epigenetic drugs can affect the expression of neuronal specific genes. In this study, we have shown that DAC is a *CYP46A1* inducer, which can further potentiate the TSA-dependent increase in *CYP46A1* gene expression, by a DNA methylation independent mechanism, inducing a decrease in Sp3/ HDAC binding to the promoter of this neuronal specific gene. Furthermore, affecting the expression of the Sp3 transcription factor, specifically in neuronal cells, DAC might not only affect brain cholesterol metabolism, but potentially affect the expression of many other neuronal genes.

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CONCLUDING REMARKS

The original studies presented in this thesis elucidate, for the first time, key mechanisms and factors involved in the regulation of the human *CYP46A1* gene. We show that the Sp transcription factors are key regulators of *CYP46A1* expression and are involved in the DAC-mediated derepression of *CYP46A1*, in a proximal promoter methylation-independent mechanism. Furthermore, our results suggest that progenitor cells eliminate cholesterol in the form of 27OHC, while neurogenesis induces a shift to the cholesterol 24-hydroxylase-dependent elimination pathway.

In the first part of our studies we have cloned and characterized the human *CYP46A1* promoter. We found it to be highly GC rich and identified a region spanning from nucleotides -236/-64 that encompasses four essential GC boxes, which is indispensable for basal expression of this TATA-less gene. In addition we have shown that the Sp transcription factors Sp1, Sp3, and Sp4 regulate the cholesterol 24-hydroxylase gene expression, and that there is an increase in the ratio of (Sp3+Sp4)/ Sp1 proteins bound to the proximal promoter Sp-response elements *in vitro*, when using nuclear extracts prepared from primary rat cortical neurons. Actually, the hypothesis that a substitution of Sp4 by Sp1 would be a mechanism of gene regulation in neurons, had previously been put forward by Steve Barger (Mao *et al.* 2006). By performing ChIP analysis we were able to determine that indeed Sp1 is dissociated from the *CYP46A1* proximal promoter region after differentiation of human NT2 cells into post-mitotic neurons, however the levels of both Sp3 and Sp4 remain unchanged. Thus, although we failed to see a substitution of Sp1 by Sp4, we confirmed our *in vitro* findings, by detecting Sp3 and Sp4 as the major proteins bound to the proximal promoter of *CYP46A1* in human post-mitotic neurons.

The fact that the different Sp proteins bind to similar response elements and can modulate transcription by protein-protein interactions, raises the question of whether the displacement of Sp1 can cause a shift in the conformation of the promoter region, that leads to the differential recruitment of co-activators/ co-repressors or even components of the core initiation machinery. Indeed, it was recently described that a switch in specific subunits of the ATP-dependent chromatin remodeling complexes must be exchanged during the transition from neuronal progenitors to post-mitotic neurons. This switch involves the exchange of the subunits BAF45a and BAF53a of the neural-progenitor-specific Brg/ Brahma associated factor (BAF) complex, for the homologous BAF45b and BAF53b subunits within neuron-specific BAF (nBAF) complex in post-mitotic neurons and preventing the subunit switch impairs neuronal differentiation (Lessard *et al.* 2007). The BAF53b subunit and the nBAF complex have been shown to be essential for an

evolutionarily conserved program of post-mitotic neural development and dendritic morphogenesis (Lessard *et al.* 2007, Wu *et al.* 2007). Since, Sp1 has been shown to interact with the BAF complex *in vivo* and to increase the BAF-mediated activation of the interferon-stimulated gene factor 3 promoter (Liu *et al.* 2002), one may speculate that such a switch, along with the dissociation of Sp1 transcription factor from the promoter, could be important in prompting the expression of neuron-specific genes, such as *CYP46A1*.

Bioinformatic analysis has shown the existence of two putative RE1 sites upstream of the *CYP46A1* proximal promoter (unpublished data). REST has been shown to inhibit the Sp1-dependent transcriptional activation of target genes, in non-neuronal cells (Myers *et al.* 1998, Plaisance *et al.* 2005, Park *et al.* 2007). Furthermore, it has been widely described that REST interacts with several chromatin-modifying proteins such as HDACs (reviewed in Ooi & Wood 2007), and our group and others have shown the importance of HDACs on the repression of *CYP46A1* transcription in non-neuronal cells (Shafaati *et al.* 2009, Nunes *et al.* 2010). As such, one may speculate that the repressor REST can play a role in controlling the tissue-specific expression of this gene.

Since replacement of Sp1 by Sp4 might have important effects on neuronal gene expression, and consequently on the differentiation of post-mitotic neurons, we have assessed the binding pattern of Sp transcription factors to the promoters of other Sp-regulated genes expressed in neurons. In the reelin *locus* we could indeed detect a substitution of Sp1 by Sp4, at the proximal promoter region; however, we failed to detect any conserved binding pattern of Sp proteins, after neuronal differentiation, at the promoters of the other genes analyzed. For instance, in the GRIN1 promoter, after neuronal differentiation, we no longer detected Sp4 association, while in the MOR promoter we observed the recruitment of Sp1. Although, in contrast with what initially hypothesized, these results are not completely unexpected, since all of the genes studied have different promoter arrangements with regard to the Sp response elements (Wendel & Hoehe 1998, Liu *et al.* 2003, Mejia-Guerra & Lareo 2005, Chen *et al.* 2007), and the majority of studies point to the major importance of the promoter context in Sp-dependent tissue-specific gene expression (Faraonio *et al.* 1994, Liu *et al.* 2003, Ross *et al.* 2002b, Yamaguchi *et al.* 2010). Indeed, our results reinforce the notion that binding of Sp proteins to DNA greatly depends on promoter context suggesting that, in the neuronal chromatin environment, Sp1-dependent transactivation of specific neuronal promoters is

most likely to occur, despite the significant decrease in Sp1 levels after neuronal differentiation.

Brain cholesterol elimination depends on the neuronal expression of CYP46A1 and the lack of a cellular model that expresses high levels of this enzyme has limited the insight into how human post-mitotic neurons handle cholesterol. We have shown that cholesterol synthesizing enzymes are decreased in NT2N cells, and most importantly that the cholesterol 24-hydroxylase expression is up-regulated, which reflects what is thought to occur *in vivo*. Although the complexity of brain structure and diversity of cell populations cannot be mimicked by an *in vitro* system, the NT2 cell model is a widely accepted surrogate of mature neurons of the human CNS (Llanes *et al.* 1995, Freemantle *et al.* 2002, Deb-Rinker *et al.* 2005). Furthermore, the use of original human tissues is limited because of availability and ethical concerns, taking into account the differences in cholesterol homeostasis between rodents and humans (Dietschy & Turley 2001, Dietschy & Turley 2004, Heverin *et al.* 2005) and given our results, we believe that the NT2 cell model can be a valuable *in vitro* model to study neuronal cholesterol metabolism and to test potential therapeutic agents that can modulate *CYP46A1* expression.

To our knowledge this was the first time that CYP46A1 protein and 24OHC have been detected in human neuronal cells in culture. Moreover, concomitantly with the increase in CYP46A1 protein, we observed a decrease in CYP27A1 protein levels, and an increase in CYP39A1 and CYP7B1, the enzymes responsible for metabolizing 24OHC and 27OHC. Interestingly, we have also shown that RA-induced transition from progenitors to fully differentiated neural phenotype, induces a shift in the cholesterol catabolic pathway utilized by these cells, suggesting that progenitor cells eliminate cholesterol in the form of 27OHC, while post-mitotic neurons depend on cholesterol 24-hydroxylation. *CYP46A1* expression increases after birth reaching steady-state levels that are maintained through adulthood. However, nothing is known about CYP27A1 expression in the developing brain, and it would be interesting to investigate if the shift in the utilization of the sterol-hydroxylases also occurs *in vivo*.

The lack of enzyme-product correlation observed when we compared the changes in 24OHC and 27OHC levels with the changes in CYP46A1 and CYP27A1 protein levels, led us to hypothesize that CYP46A1 can further metabolize 27OHC. It has been shown that CYP46A1 presents broad substrate specificity and that the substrate-binding pocket of this enzyme is large and capable of accommodating structurally diverse substrates of different sizes (Mast *et al.* 2003). Moreover, CYP46A1 has been shown to further

metabolize 24OHC into 24,27-dihydroxycholesterol, binding to 24OHC with an higher affinity than to cholesterol (Mast *et al.* 2003). Our results do not show an increase in 24OHC concomitant with the increase in CYP46A1 protein, but show a correlation between the decrease in 27OHC and the increase in CYP46A1. Therefore, it is possible that, not only 24OHC is converted to 24,27-dihydroxycholesterol, but also that 27OHC in excess could be further metabolized by CYP46A1, in post-mitotic neurons. Indeed, we are currently addressing this issue by over-expressing CYP46A1 in human embryonic kidney 293 cells, and trying to detect an increase in the levels of 24,27-dihydroxycholesterol, after addition of exogenous 27OHC.

If we can prove that 27OHC is a substrate for CYP46A1, this has important physiological consequences. Although a flux of 27OHC from the plasma to the brain has been demonstrated, the levels of this oxysterol in the brain are extremely low (Heverin *et al.* 2004, Heverin *et al.* 2005). 27OHC has been shown to be metabolized by CYP7B1 to 7α-hydroxy-3-oxo-4-cholestenoic acid *in vitro*, which can rapidly traverse an *in vitro* model of the BBB (Meaney *et al.* 2007). Furthermore, by measuring the concentrations in the internal jugular vein and in the brachial artery of healthy volunteers, a net flux of this metabolite from the brain into blood circulation has been demonstrated, which is consistent with this being an important terminal metabolite of 27OHC in the brain. However, the magnitude of the flux of 7α-hydroxy-3-oxo-4-cholestenoic acid from the brain is much lower than could be expected if this was the only metabolite of 27OHC leaving the brain (Meaney *et al.* 2007). If it is confirmed that CYP46A1 metabolizes 27OHC, it is possible that this activity towards 27OHC is responsible for the discrepancy observed by Meaney and co-workers (2007).

We have previously shown that TSA, an HDAC inhibitor can up-regulate *CYP46A1* expression, by affecting the histone acetylation levels at the proximal promoter (Nunes *et al.* 2010). Moreover, bioinformatic analysis of the 5' flanking region predicted that the proximal promoter region of *CYP46A1* is located in a CpG island. We have hypothesized that a cross-talk between DNA methylation and HDAC/ HAT dependent-chromatin modifications could be involved in the cell-specific expression of this gene. Treatment of neuroblastoma cells with the demethylating agent DAC, showed a time- and dose-dependent derepression of *CYP46A1*. Furthermore, pre-treatment with DAC, synergistically enhanced the TSA mediated up-regulation, pointing to the possibility that indeed an epigenetic program could be involved for the tissue-specific expression of *CYP46A1*. Surprisingly, bisulfite sequencing analysis demonstrated that the core

promoter is completely unmethylated, even in human tissues where *CYP46A1* is not expressed, which suggests that DNA methylation of the proximal promoter does not play a role in the regulation of *CYP46A1* tissue-specific expression. Interestingly, Sp1 binding to DNA regulatory elements has been shown to protect CpG islands from methylation (Brandeis *et al.* 1994, Macleod *et al.* 1994). Since ChIP analysis demonstrated that Sp1, Sp3 and Sp4 are bound to the proximal promoter in untreated cells, where CYP46A1 mRNA is barely detected, it is possible that *CYP46A1* proximal promoter is protected from CpG methylation by this mechanism. Interestingly, DAC has been reported to induce the expression of other unmethylated silenced Sp-regulated genes (Kim *et al.* 2003, Yokota *et al.* 2004, Scott *et al.* 2006). Nevertheless, we cannot exclude that methylation of an enhancer or other regulatory element not yet identified, outside of the *CYP46A1* core promoter, can interfere with *CYP46A1* expression, or that DNA demethylation may reactivate an unidentified transcription factor that controls *CYP46A1* expression.

DAC has been approved by the Food and Drug Administration to treat myelodysplastic syndrome and has been shown to effectively traverse the BBB. However, the characterization of the molecular mechanisms by which DAC can affect the expression of neuronal specific genes in a DNA methylation-independent manner, is not well known. We observed that DAC treatment decreased Sp1 and Sp3 protein levels. Interestingly, DAC has been shown to affect the levels of specific proteins by inducing degradation by the proteasomal pathway (Cecconi *et al.* 2009, Patel *et al.* 2010). Moreover, we observed a decrease in the binding activity of Sp3 and HDAC1 and 2, to the +1 region of the *CYP46A1 locus*. This decrease in Sp3 is in agreement with previous results from our laboratory (Nunes *et al.* 2010), where we show that a decrease of Sp3 induced by TSA, favors the detachment of both HDAC1 and 2 from the *CYP46A1* proximal promoter. In fact Sp3 has been demonstrated to physically interact with HDACs (Sun *et al.* 2002, Zhang & Dufau 2002). Loss of Sp3 binding induced by DAC, which may be required for HDAC association, may facilitate the TSA-induced derepression of *CYP46A1*, suggesting that DAC can directly induce regional chromatin remodeling, independently of its effects on DNA methylation. It would be of great interest to understand if, in neuronal cells, DAC treatment induces Sp1 and Sp3 degradation by the proteasomal pathway, and if these effects associated with inhibition of HDAC activity are sufficient to induce *CYP46A1* expression, or if additional neuron-specific factors are also necessary to up-regulate this gene.

The fact that, so far the only compounds known to up-regulate the expression of the *CYP46A1* gene are TSA and DAC, drugs that can modify the epigenome, raises the question of the use of these agents in the clinic. Several studies have shown that only a small subset of genes display altered expression profiles after treatment with this type of drugs (Marks *et al.* 2000, Glaser *et al.* 2003, Karpf *et al.* 2004, Shafaati *et al.* 2009). However, it is of the utmost importance to assess both *in vitro* and *in vivo* the various molecular players and pathways involved in the CNS proper functioning that are affected by these drugs, both in normal and in different pathophysiological conditions. For instance, although valproate is widely used as a mood stabilizer, a recent clinical trial demonstrated significant worsening of agitation and aggression in AD patients receiving valproate when compared to placebo (Herrmann & Lanctot 2007). Whether the pharmacological manipulation of the epigenome will provide successful therapeutic outcomes remains unclear, particularly because current treatments are unable to modulate specific genomic regions, and this may increase the risk of off-target responses.

Our results also raise another unanswered question, regarding the correlation between an alteration in the ratio of Sp proteins that are bound to the *CYP46A1* promoter and the up-regulation of this gene. Indeed, after neuronal differentiation Sp1 is dissociated from the promoter, whereas after treatment with DAC it is Sp3 binding that is decreased. Although Sp4 binding remains unchanged, our results imply that the shift in the ratio of the Sp proteins bound to the promoter is not the single event leading to an increase in *CYP46A1* expression. In agreement, specific knockdown of Sp3 with siRNAs was not sufficient to potentiate TSA-mediated upregulation of *CYP46A1* (Nunes *et al.* 2010). Sp proteins suffer a wide range of PTMs, which modulate the activity of these transcription factors (reviewed in Wierstra 2008). Phosphorylation is one of the major modifications described to regulate Sp proteins transcriptional activity. In fact, Sp1 has been shown to be phosphorylated by at least nine kinases, and two phosphatases have also been shown to regulate its phosphorylation levels. Interestingly, both DAC and TSA are known to activate signaling pathways, which modulate the activity of specific kinases and phosphatases (Brush *et al.* 2004, Wang *et al.* 2008a, Simboeck *et al.* 2010, Takano *et al.* 2010), which in turn can target Sp proteins. Furthermore, it has been shown that TSA can induce changes in Sp proteins PTMs, without affecting Sp binding to the promoter of target genes (Zhang *et al.* 2006, Zhang *et al.* 2008). Moreover, it has been shown that Sp3 is regulated by SUMOylation (Sapetschnig *et al.* 2002, Ross *et al.* 2002a) and this modification leads to local heterochromatic gene silencing (Stielow *et al.* 2008).

SUMOylation has been shown to be important in both neuronal development (Shalizi *et al.* 2006) and in neurological disorders such as AD (reviewed in Martin *et al.* 2007). In order to thoroughly dissect the underlying mechanisms that lead to an increased *CYP46A1* expression it would be of great interest to study the signaling pathways activated by DAC and TSA treatment and during neuronal differentiation, which can induce changes in Sp transcription factors PTMs and differentially regulate their transcriptional activity.

In conclusion, the results presented in this thesis are a significant contribution to the definition of the regulatory circuits that control human *CYP46A1* expression and may prove useful in unraveling potential therapeutic agents and approaches that can modulate *CYP46A1* expression, and thus control brain cholesterol metabolism in different pathophysiological conditions.

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